Impact of Intrapartum Oral Azithromycin on *Staphylococcus aureus* Colonisation and Staphylococcal Antibiotic Resistance Among Women and Their Babies in The Gambia

Thesis

Impact of intrapartum oral azithromycin on *Staphylococcus aureus* colonisation and staphylococcal antibiotic resistance among women and their babies in The Gambia

Thesis Submitted to the Open University, UK in fulfilment of the requirement of the Degree of Doctor of Philosophy (PhD) in the field of Science

Abdoulie Bojang (F5736605) - BSc (Hons) MSc

Affiliated Research Centre:	Medical Research Council Unit The Gambia at the London School of Hygiene & Tropical Medicine (MRCG@LSHTM), The Gambia
Director of studies:	Professor Anna Roca BSc MSc PhD MRCG@LSHTM, Fajara The Gambia
Supervisor 1:	Professor Ben Howden MD PhD Microbiological Diagnostic Unit Public Health Laboratory, Department of Microbiology and Immunology, University of Melbourne at The Doherty Institute for Infection and Immunity, Melbourne, Victoria, Australia
Supervisor 2:	Dr Christian Bottomley BSc MSc PhD Medical Research Council Tropical Epidemiology Group, London School of Hygiene & Tropical Medicine, London, UK

Date of submission: 29th February 2020

ABSTRACT

Maternal and neonatal infections are a major public health problem leading to high morbidity and mortality in middle and low-income countries. There is a need for effective interventions suitable for these countries to reduce the high burden of disease.

Intrapartum azithromycin is a potential intervention to reduce maternal and neonatal infections and their associated deaths. PregnAnZI-1, the first intrapartum oral azithromycin trial was a Phase III, double-blind, placebo controlled randomized clinical trial in which 829 women in labour were randomized to receive either a single dose of 2g of oral azithromycin or placebo (ratio 1:1). The trial was conceived as a pilot study to determine the impact of the intervention on maternal and neonatal bacterial colonization, as a necessary step towards sepsis. Nasopharyngeal swabs (NPS), breast milk (BM) and vaginal swabs (VS) were collected from study women and /or their babies during the first 4 weeks of follow-up (day 0, day 3, day 6, day 8, day 14 and day 28). Additionally, NPS were also collected from study children in a survey conducted 12 months following the trial. During the trial follow up period (birth to day 28), prevalence of carriage of *S. aureus*, *S. pneumoniae* and group B Streptococci decreased significantly in the intervention arm, whilst prevalence of azithromycin resistant *S. aureus* increased among mothers or their babies exposed to azithromycin compared to the placebo group.

The aims of my PhD investigations are to determine the long-term impact of such intervention on *S. aureus* colonisation and antimicrobial resistance; molecular epidemiology of azithromycin resistant *S. aureus* and the prevalence of nasopharyngeal macrolide resistance genes following intrapartum oral azithromycin intervention.

In the survey conducted 12 months following the main trial, a total of 461 NPS were screened for prevalence of carriage and azithromycin resistance for *S. aureus* and *S. pneumoniae*. As expected there was no difference in the prevalence of nasopharyngeal carriage of *S. pneumoniae* or *S. aureus* between children whose mothers had been exposed to azithromycin during labour and those whose mothers had not (85.0% vs 82.1%; odds ratio [OR], 1.23 [95% confidence interval, .73-2.08] for *S. pneumoniae* and 21.7% vs 21.3%; OR, 1.02 [95% CI, .64-1.64] for *S. aureus*). Also, prevalence of azithromycin-resistant *S. pneumoniae* (1.8% vs 0.9% p= 0.384) and *S. aureus* (3.1% vs 2.6% p= 0.724) were not different between children from the azithromycin and placebo arms, respectively. The intervention did not induce antibiotic resistance to other antibiotics commonly used in Africa for these two bacteria.

A random selection of 7 mothers and 10 babies from both arms of the trial, who carried *S. aureus* resistant to azithromycin in the nasopharynx at day 3 and day 28 post-intervention were included in the second investigation undertaken as part of this PhD. A total of 66 *S. aureus* isolates underwent genomic investigation. Seven *S. aureus* sequence types (STs) were identified. ST5 predominated in the placebo arm (73.0% versus 49.0%, p = 0.039) and ST15 in the azithromycin arm (27.0% versus 6.0%, p = 0.022). Among azithromycin-resistant isolates, *msr(A)* gene was the main macrolide resistance gene (n = 36, 80%) and was found to be located on a multidrug resistant (MDR) plasmid. The intervention appeared to select for the *ermC* gene as 36% of resistant *S. aureus* isolates from mothers and babies exposed to antibiotic carried the gene as oppose to 0% in the placebo arm (p< 0.001).

Having established that the genetic determinants for *S. aureus* macrolide resistance were predominantly *msr(A)* and *ermC*, I assessed the effect of intrapartum oral azithromycin on these genes in my final PhD investigation as they were found to be located on mobile genetic

elements that can transfer horizontally between bacteria. PCRs were performed on 936 NPS from 312 children at three different time points (birth, day 28 and 12 months). At birth, prevalence of *msr(A)* gene was similar in both arms, higher in the azithromycin arm by day 28 (60.7% vs. 29.9% in the azithromycin and placebo arms, respectively; OR, 3.61 [95% CI, 2.20-5.93]) and again similar between arms at 12 months. Prevalence of *ermC* followed a similar pattern, with differences between arms only apparent at day 28 (63.9% vs. 45.9% in the azithromycin and placebo arms, respectively; OR, 2.09 [95% CI, 1.29-3.37]).

In conclusion, the effectiveness of intrapartum oral azithromycin prophylaxis in decreasing carriage of bacteria associated with both maternal and neonatal infections had been established. Emergence of azithromycin resistant *S. aureus* shortly (28 days) following oral azithromycin exposure was of concern. However, in the long term (12 months), neither the increased azithromycin resistant *S. aureus* nor the increased nasopharyngeal macrolide resistance genes observed shortly following the intervention persisted among mothers or their new-borns. Hence, supporting the use of intrapartum oral azithromycin prophylaxis as a means to reduce maternal and neonatal infections in The Gambia and potentially other developing countries worldwide.

ACKNOWLEDGEMENT

All praises be to Allah the lord of the worlds for sparing my life to undertake PhD studies.

I would like to thank the Medical Research Council Unit The Gambia at the London School of Hygiene and Tropical medicine for awarding me a full 4-year PhD scholarship to undertake PhD studies which has been a dream come through.

I would also like to express deep gratitude to Professor Anna Roca director of my PhD studies for her guidance, enthusiastic encouragement and useful critiques of my research work. I am very grateful to my external supervisor Professor Ben Howden for his guidance and also for providing financial assistance for the molecular and bioinformatics projects of my PhD. My sincere thanks go to the following supervisors or advisers for their invaluable support during the course of my PhD studies: Professor Umberto D'Alessandro, Drs Christian Bottomley, Ousman Secka, Sarah Baines, and Romain Guerillot.

I am extremely grateful to the PregnAnZI -1 field and laboratory staff for helping with the sample collection and processing results of which became the basis of my PhD.

Finally, I wish to thank my wife and entire family for their understanding as I had to stay away from the family in order to concentrate on my studies and only get to see them during weekends. My mum and dad believe in the power of supplication (Dua) and always prayed and encouraged me throughout my studies.

DEDICATION

This work is dedicated to my dad Alh. Mawdo Bojang, mum Aja Manyima Marrie, aunty Aja Jokor Sanka Bojang, wife Ndey Jallow Bojang, children, sisters and brothers.

STATEMENT OF WORK PERFORMED BY SUPERVISORS/COLLEAGUES

- Professor Anna Roca was the director of my PhD studies. She was the PI of the PregnAnZI-1 trial were the samples came from. In addition, she assisted with the design of all of the investigations undertaken, reviewed all manuscripts from the first draft, provided funding for lab reagents/consumables and mentorship.
- Dr Bully Camara and Dr Claire Oluwalana were clinicians in the main trial (PregnAnZI-1). They assisted with the collections of samples used as part of my PhD and also contributed to writing and publications of the articles.
- Dr Ousman Secka assisted in supervising all the microbiology and some of the molecular biology work and also contributed in the publications
- Dr Christian Bottomley review all the statistical analysis and also contributed in the publication all of my articles.
- Professor Ben Howden's lab team including Professor Tim Stinear, Dr S Baines, Mr Liam Donovan, Dr R Guerillot, Ms Kerrie Stevens, Dr C Higgs, Dr MB Schultz, Dr SA Gonçalves, Professor T Seemann Australia assisted with the library prep and whole genome sequencing of *S. aureus* isolates, provided training in bioinformatics analysis, phylogenetic tree construction using maximum likelihood method. In addition, I received training in other microbiology techniques including the use of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI TOF MS) and VITEK 2.

PEER-REVIEWED PUBLICATIONS INCLUDED IN THE THESIS

1. **Bojang A,** Baines SL, Camara B, Guerillot R, Marquez RS, Secka O, D'Alessandro U, Bottomley C, Howden BP, Roca A Impact of oral intrapartum azithromycin on the nasopharyngeal Staphylococci macrolide resistome of babies: a randomised control trial *Clin Infect Dis.* 2020 May 23; ciaa609. doi: 10.1093/cid/ciaa609. Online ahead of print

2. **Bojang A**, Baines SL, Donovan L, Guerillot R, Stevens K, Higgs C, Bottomley C, Secka O, Schultz MB, Gonçalves da Silva A, Seemann T, Stinear TP, Roca A, Howden BP Genomic investigation of *Staphylococcus aureus* recovered from Gambian women and new-borns following an oral dose of intrapartum azithromycin. *J Antimicrob Chemother.* 2019 Aug

3. **Bojang A**, Camara B, Jagne Cox I, Oluwalana C, Lette K, Usuf E, Bottomley C, Howden BP, D'Alessandro U, Roca A. Long-term Impact of Oral Azithromycin Taken by Gambian Women During Labour on Prevalence and Antibiotic Susceptibility of *Streptococcus pneumoniae* and *Staphylococcus aureus* in Their Infants: Follow-up of a Randomized Clinical Trial. *Clin Infect Dis.* 2018 Sep

Contents

CHAPTER 1

1.0 LITERATURE REVIEW

1.1 Epidemiology of *Staphylococcus aureus* disease

S. aureus is a major human pathogen with the ability to infect a range of tissues of the human body. Infections caused by *S. aureus* can be divided into three general categories: (a) life-threatening systemic conditions such as; sepsis, bacteraemia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome; (b) Skin and soft tissue infections (SSTIs) e.g. impetigo, boils, cellulitis, folliculitis, carbuncles, scalded skin syndrome, abscesses and; (c) toxinoses e.g. food poisoning [1].

Among the range of infections caused by *S. aureus,* bacteraemia is often more associated with higher morbidity and mortality. In the last 20 years, due to increased frequency of invasive procedures, increased numbers of immunocompromised patients, and increased resistance of *S. aureus* strains to available antibiotics, incidence of *S. aureus* bacteraemia and its complications has increased sharply [2-4]. Globally, *S aureus* bacteraemia is one of the most common bloodstream infection [5, 6] and the burden varies between developed [7-9] and developing [10-12] countries with developing countries bearing the highest burden.

1.2 Burden of *S. aureus* bacteraemia in developed countries

S. aureus disease ranks very high in the public-health agenda of developed countries as a result of high burden of disease associated with the bacteria. In the United States, *S. aureus* is the most frequently occurring bacterial pathogen among clinical isolates (blood, samples from lower respiratory tract, skin and soft tissue) from hospital inpatients and the second

most prevalent bacterial pathogen among clinical isolates from outpatients [13]. Annually, 490,000 hospitalizations; 93,000 cases of bacteraemia; and 35,000 cases of sepsis and/or endocarditis are due to *S. aureus* [14, 15]. In Denmark, among all age groups, incidence of *S. aureus* bacteraemia between 1995 and 2008 was reported to be 22.7 cases per 100,00 person years. The figure increased to about 30 cases among adults aged more than 21 years and then increased further to 494 cases among HIV infected individuals age ≥ 16 year [16-18]. Incidence rates in Iceland, Finland, Sweden and Netherlands are fairly similar ranging between 14 to 33 cases per 100,000 person years [19-22].

1.3 Burden of *S. aureus* bacteraemia in Sub Saharan Africa

In developing countries such as those in the Sub Saharan Africa, *S. aureus* disease does not appear to rank very high in the public-health agenda even though these countries bear the greatest burden of diseases due to the bacteria [23].

Bloodstream infections are among the leading causes of morbidity and mortality in people of all ages in sub-Saharan Africa [24]. In Senegal, high rate of *S. aureus* bacteraemia was reported among patients between January 1996 and December 2002 in a hospital in the capital Dakar with even high rate of Methicillin-resistant *Staphylococcus aureus* (MRSA) bacteraemia among nosocomial bacteraemia [25]. Among older children age <15 years in Mozambique, incidence of *S. aureus* bacteraemia was reported to be 48 cases per 100,000 per years [26]. In Kenya, incidence of *S. aureus* bacteraemia per 100,000 person years among children age less than 5 years between 1998 and 2002 was reported to be 27 cases[27]. Among the same age group, a higher incidence of 630 cases per 100,000 person years of *S. aureus* bacteraemia was reported among Ghanaian children between 2007 and 2009 [28]. It

appears at least from the above data that incidence of *S. aureus* bacteraemia varies extensively between countries in Africa with highest incidence in western Africa compared to eastern, western and southern African countries. Similarly, among neonates, *S. aureus* bacteraemia was highest in western Africa accounting for more than 77% of all Gram-positive causes of bacteraemia or sepsis compared to 59% in central Africa, 54% in eastern Africa and 37% in Southern Africa [29].

1.4 Burden of *S. aureus* bacteraemia in The Gambia

In the Gambia, *S. aureus* is the main cause of bacteraemia in all age groups and the most prevalent cause of neonatal sepsis [11, 30, 31]. More than 15 years ago, *S. aureus* was the second most common cause of bacteraemia among all ages groups accounting for about 18.3% of bacteraemia cases with a positive blood culture [30]. Incidence among children age less than 5 years was more than double to that reported in Kenya but far less than the 638 cases per 100, 000 reported in Ghana [28]. Prior to introduction of pneumococcal conjugate vaccine into the Gambian immunisation program in August 2009, *Streptococcus pneumoniae* was the leading cause of invasive bacterial disease leading to bacteraemia [30]. However, following the introduction of the pneumococcal conjugate vaccine, *S. aureus* became the leading cause of invasive bacterial diseases leading to high morbidity and mortality [24]. Among all age groups, the proportion of *S. aureus* bacteraemia increased post vaccination from 16.9% to 27.2%; becoming the most prevalent bacteria associated with invasive bacterial infections in The Gambia [24] **Figure 1**.

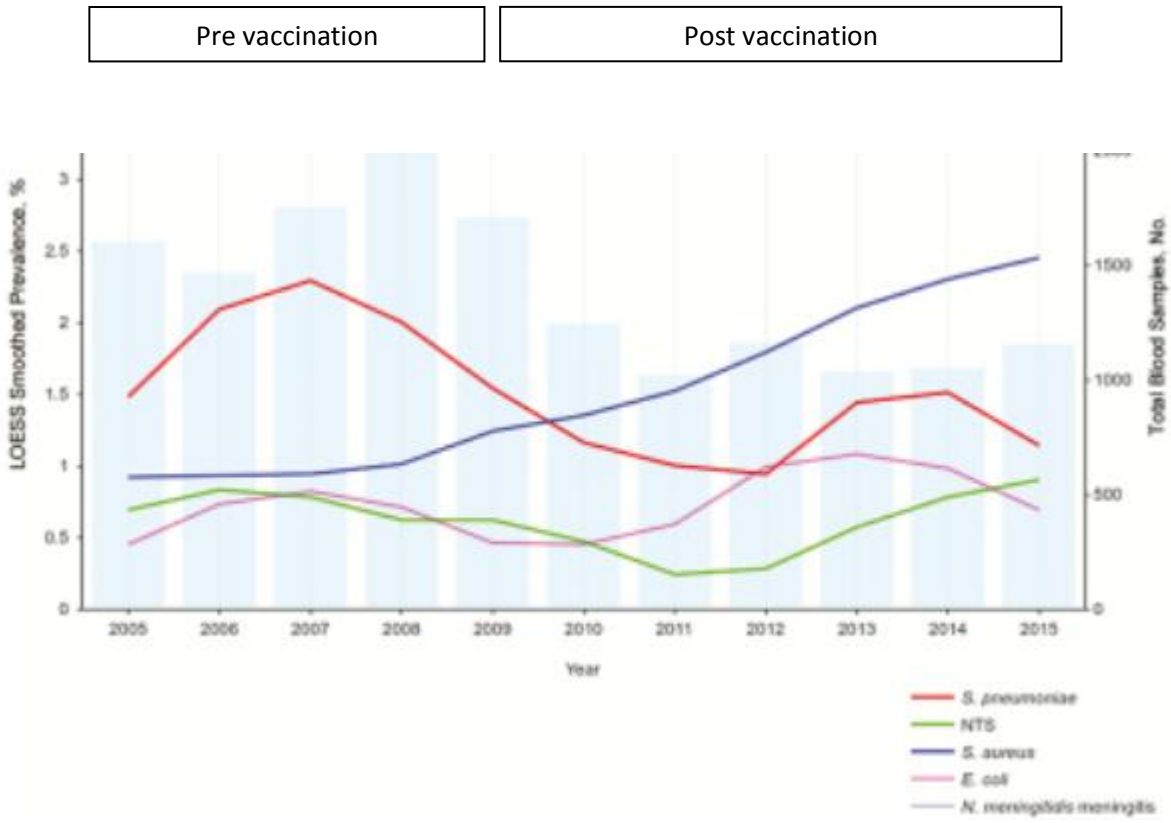


Figure 1 Annual trends in major bacterial pathogens associated with invasive bacterial infections in The Gambia, 2005–2015, in relation to the introduction of pneumococcal conjugate vaccines adapted from [24]. Prevalence of *S. aureus* bacteraemia (blue line) increased exponentially post vaccination.

Incidence of *S. aureus* bacteraemia among children < 5 years of age in 2 regions in the eastern part of The Gambia during 2008–2015 was reported to be 78 cases per 100,000 person-years. Among neonates, the number of cases increased substantially to 2,080 cases per 100,000 person-years [11]. In the eastern region of The Gambia, there exist a health and demographic surveillance system that covers a population of about 170,000 in 219 settlements. Studies conducted in these areas report incidence using the total population as the denominator whereas in the western regions, only laboratory based data are available.

1.5 *Staphylococcus aureus* carriage

Asymptomatic carriage of *S. aureus* often precedes disease and carriage *S. aureus* species are responsible for transmission in the community and also represent circulating strains in the community [32-34]. The carriage of *S. aureus* varies depending on a number of factors including geographic location, age, gender, ethnicity and body niche [35]. The most frequent site colonised in adult population includes the nares, throat and perineum. Other sites such as the skin, vagina and intestine are also frequently colonised [35, 36]. **Figure 2** shows the various body parts colonised by *S. aureus* [37]. Colonization offers a reservoir from where the pathogen can access the bloodstream for example when breaches appear in host defence systems but infections can also occur without nasal colonization [38]. The association of nasal carriage and *S. aureus* disease has been reported as early as 1931 [39]. Subsequently, a number of other studies also confirmed the association of *S. aureus* nasal carriage and disease [40-42]. The above findings were supported by the fact that the nasal *S. aureus* strain and the infecting strain share the same phage type or genotype [42]. Furthermore, decolonisation

of the nose by applying anti staphylococcal drug have also been shown to temporarily prevent *S. aureus* infection [43].

Carriage has been reported to be higher in young children [32], men [44], hospitalised patients and a number of other patient groups, including patients with diabetes mellitus [45], those with *S. aureus* skin infection [46], and HIV-infected patients [37]. In some individuals, carriage strains may remain resident for an extensive long period even for a few years [47] but it is not clear how nasal carriage is established and maintained [37]. In sub Saharan Africa, nasal carriage of *S. aureus* varies extensively. In Ethiopia, prevalence of *S. aureus* nasal carriage among pre-School children was reported to be 13% [48]. Among Tanzanian children under the age of 5 years, nasal carriage of *S. aureus* was reported to be 40% [49] and 22.1% of Ghanaian children < 15 years of age on admission were reported to carry *S. aureus* in the nasal cavity [50].

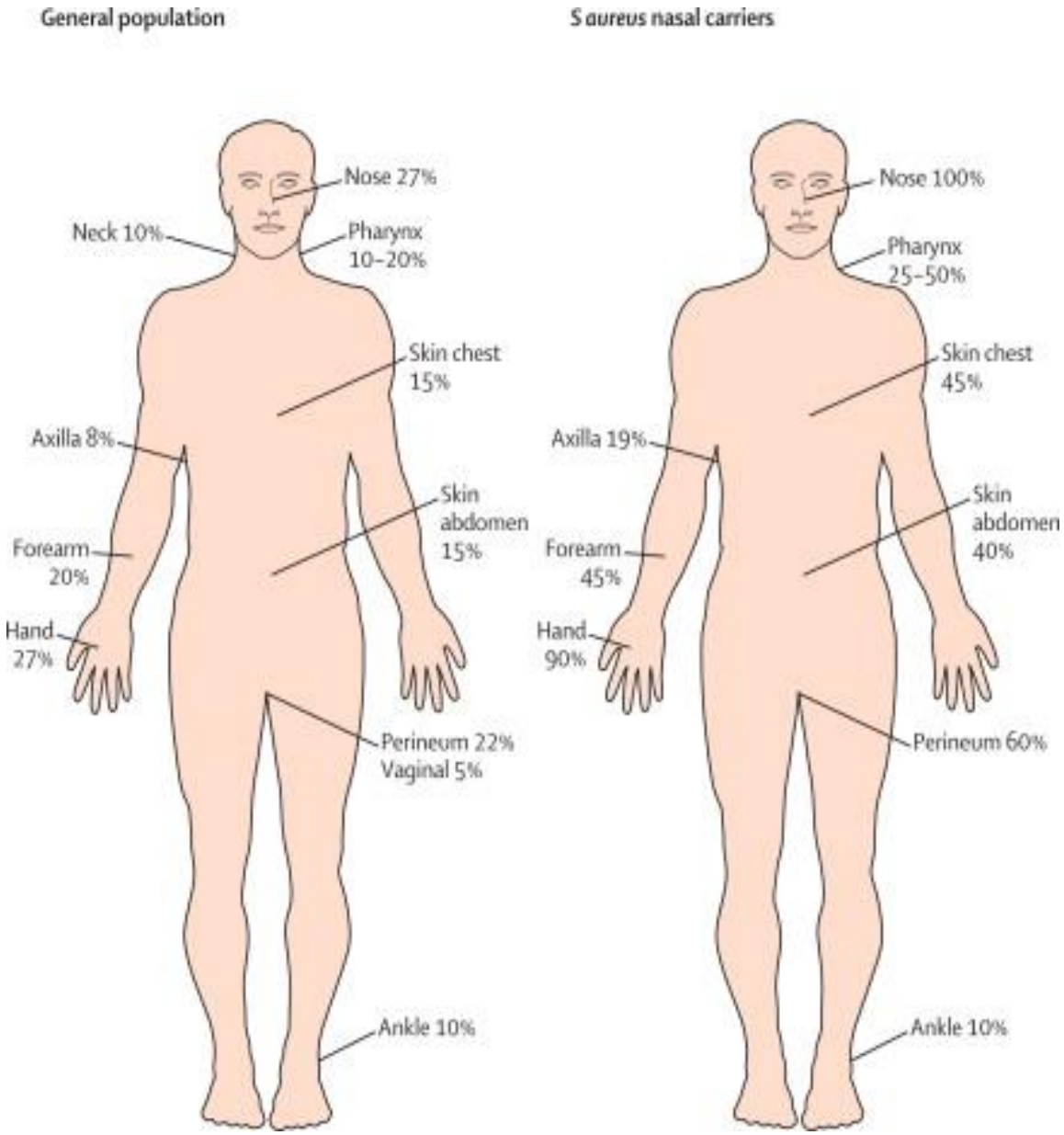


Figure 2. The various body parts colonised by *S. aureus*. Colonisation rates per body site increased among nasal carriers reproduced from [37].

1.6 *S. aureus* carriage in The Gambia

In The Gambia, a number of studies described carriage of *S. aureus* from various body sites in different age groups [51-53]. In a cohort of Gambian women sampled at delivery and other time points during the follow up period, up to 71% of the women were found to carry *S. aureus* at some point in either the nasopharynx, breast milk or vaginal [54]. At least at one time point, nasopharyngeal, breast milk and vaginal tract carriage of *S. aureus* were found to be 51.5%, 35.2% and 24.5% respectively [54]. Among older children age 5-10 years nasopharyngeal carriage was reported to be up to 25.9% [55]. Nasopharyngeal carriage among children age 1 year or less immunised with the pneumococcal conjugate vaccine (PCV-7) reported 65% and 33.6% oropharyngeal and nasopharyngeal carriage respectively [53]. Among new-borns sampled during the neonatal period (0-28 days), *S. aureus* nasopharyngeal carriage ranged between 50-63% [52, 56]. Neonatal nasopharyngeal carriage of *S. aureus* was found to be significantly associated with maternal carriage in the breast milk, vaginal tract and nasopharynx [54].

1.7 Transmission

S. aureus appears to successfully colonise almost all parts of the human body and rates of colonisation increased for each site among nasal carriers [37]. As a result of the ubiquitous nature of *S. aureus* carriage, various modes of transmission occur both within the community, between mother and child as well as in hospital wards.

1.8 Community transmission

In this mode of transmission, *S. aureus* is often transferred from the nose to the hand of a person, then to a surface, before being transferred via the hand to the nose of a second person [57]. Community transmission could also occur through direct skin contact between carriers and non-carriers sharing personal hygiene items, for example, razors, towels, or soaps [58]. Further, inhalation of air droplets from nasal carriers after sneezing could also result in the transfer of the bacterium especially among individuals suffering from viral infections [59, 60].

1.9 Mother to child transmission

S. aureus could also be transmitted from mother to child during the process of the baby passage through the birth canal as a result of the baby coming in contact with mother's body fluids [61]. Following delivery, and during breast feeding *S. aureus* may also transfer from mother to baby either from contaminated items or through breast milk [54, 62]. Human milk is a continuous source of bacteria for the infant gut, and both culture-dependent and microbiome studies have revealed that staphylococci, mainly CNS, constitute the dominant bacteria in this biological fluid [63]. Microbial communities in human milk collected from mothers living in 11 international sites including The Gambia, revealed *Streptococcus* and *Staphylococcus* as the core genera in milk although there exist differences within and across populations [64]. Studies also indicated that some bacteria present in the maternal gut, including staphylococci, can reach the mammary gland during late pregnancy and lactation through a mechanism involving gut monocytes [65, 66]. Although *S. aureus* and other bacteria have been detected from breastmilk in mothers participating in the PregnAnZI

trial [56] mother to child transmission could have occurred from the skin of the mothers breast or through exposure to *S. aureus* carried by the mothers at other anatomical sites. In light of the above, samples were collected with caution by cleaning the breast areola and discarding the initial milk. However, there is always the possibility that some of the bacteria was from skin although a good number of bacteria detected from the milk were not typical flora.

1.10 Hospital transmission

In hospital settings, *S. aureus* is mainly spread from person to person particularly from health care workers' hands if they are not washed or cleansed between contact with different patients. Like in the community, contaminated surfaces in the hospital or medical equipment are equally likely to be a platform of transmission of the bacteria [67, 68].

1.11 Characteristics of *Staphylococcus aureus*

1.11.1 *S. aureus* morphology

S. aureus is a Gram-positive, non-motile, non-spore forming bacterium that grows rapidly under aerobic conditions but can also grow without the need for oxygen (facultative anaerobes)[7]. Under the light microscope, *S. aureus* appears round in shape and forms irregular grape-like clusters. On blood agar, the bacteria appear as glistening, smooth, entirely raised translucent colonies that often have a golden pigment **Figure 3**.

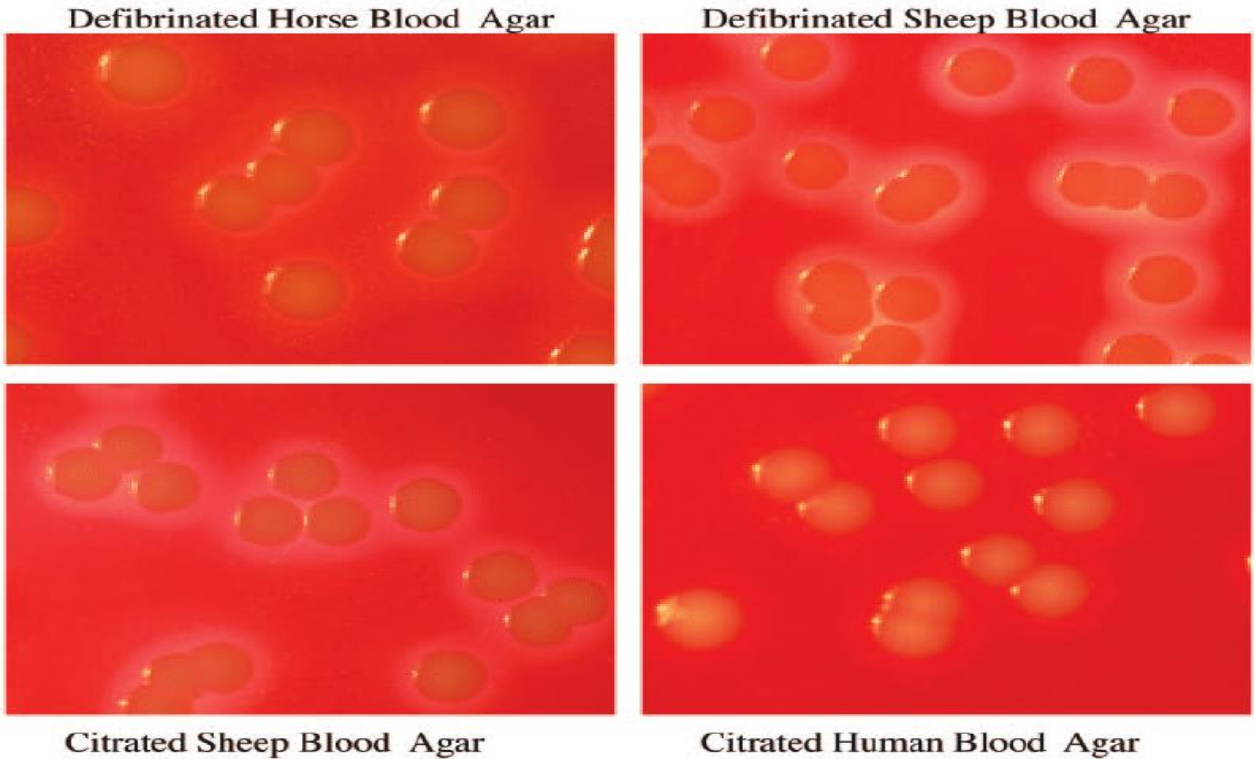


Figure 3 The colony morphology of *S. aureus* growing on different blood agar plates reproduced from [69]. The colonies are 2-3mm in diameter after 24h incubation and most strains show β-haemolysis surrounding the colonies.

S. aureus is easily identified by its ability to produce coagulase and hence clot human plasma. There are currently 2 subspecies of *S. aureus*; these are *S. aureus* subspecies *aureus* and *S. aureus* subspecies *anaerobius* [70]. Only the subspecies aureus is commonly isolated from human clinical specimens.

1.11.2 Cellular components

The components of *S. aureus* cell are an outer capsule, a thick peptidoglycan layer, cytoplasmic membrane, surface proteins and secretary proteins **Figure 4.**

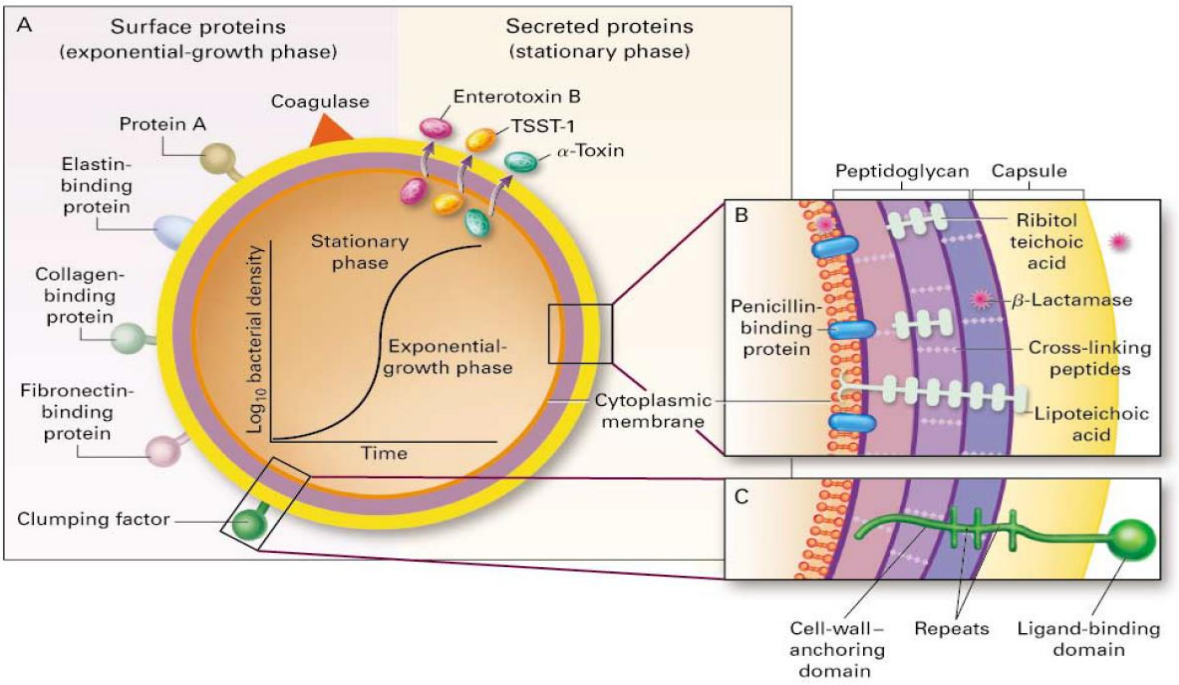


Figure 4. The structure of *S. aureus* cell reproduced from [7] **A** shows the surface and secreted proteins expressed during the exponential and stationary growth phases. **B** and **C** shows cross-sections of the cell envelope revealing detail structures of the peptidoglycan and capsule.

Like all Gram-positive bacteria, the cell wall of *S. aureus* is predominantly made of peptidoglycan. Peptidoglycan consists of alternating polysaccharide subunits of N-acetylglucosamine and N-acetylmuramic acid with 1,4-β linkages [7]. Peptidoglycans are known to have endotoxin-like activity that stimulate the release of cytokines by macrophages, activate complement system and aggregate platelets [71, 72]. The capacity of *S. aureus* to

cause certain disease conditions such as disseminated intravascular coagulation is strongly associated with the differences in the peptidoglycan structure of staphylococcal strains [73]. *S. aureus* as well as other staphylococci produce capsule. These microcapsules are mainly made of polysaccharides that are antiphagocytic [74]. So far, up to 11 different serotypes have been described however, types 5 and 8 account for 75 percent of human infections [75].

1.11.3 Surface proteins

During the exponential growth phase of *S. aureus*, a number of surface proteins are produced including protein A, coagulase, clumping factor, collagen, fibronectin and elastin binding proteins **Figure 4**. Protein A has antiphagocytic properties that are based on its ability to bind to the Fc portion of immunoglobulin [76]. The remaining surface proteins are believed to play important roles in immune evasion and during colonisation of the host as they bind to extracellular matrix molecules and have been designated microbial surface component recognising adhesive matrix molecules (MSCRAMM) [77].

1.11.4 Secretory proteins

A number of toxins produced during the stationary phase of *S. aureus* growth cycle are grouped on the basis of their mechanism of action. These toxins include enterotoxin B, toxic shock syndrome toxin-1 (TSST-1) and α toxin. Staphylococcal enterotoxin B is associated with food poisoning, non-menstrual toxic shock, atopic dermatitis, asthma, and nasal polyps in humans [78]. Only a few bacteria including *S. aureus* are known to produce superantigens (SAgs). SAgs are a family of potent immunostimulatory exotoxins. In *S. aureus* strains more than 20 distinct SAgs have been characterized and 80% of clinical strains harbour at least one

SAg gene [79]. The mode of action of SAgs involves activation of T cells leading to overproduction of cytokines resulting in systemic inflammation and shock [80]. Classically, SAgs are associated with food poisoning and toxic shock syndrome (TSS), for which toxins are the causative agent [79]. The α toxin on the other hand causes pore formation and induces proinflammatory changes in mammalian cells. These changes result in cell damage that may contribute to manifestations of the sepsis syndrome [81]. *S aureus* also produces enzymes such as protease, lipase and hyaluronidase that destroy tissue and facilitate the spread of infection to adjoining tissues. Other enzymes include β lactamase and coagulase that inactivates penicillin and convert fibrinogen to fibrin respectively [82].

1.11.5 Genome of *S. aureus*

The genome of *S. aureus* consists of a single circular chromosome of about 2.7 – 2.8 mbp. In addition, it has plasmids, mobile genetic elements [insertion sequences (IS) and transposon (Tn)], prophages and other variable elements. Genes governing virulence and resistance to antibiotics are found on the chromosome, as well as the extrachromosomal elements [83]. Location of a gene determines to a large extent whether it could be transmitted vertically or horizontally between bacteria. Virulence or antibiotic resistance genes located on the chromosome can transfer from parent to daughter cell following replication. Additionally, recombination involving the exchange of genetic material either between multiple chromosomes or between different regions of the same chromosome have also been detected in *S. aureus* genome [84]. Gene transfer can also take place independent of cell replication involving extrachromosomal genes. Key among extrachromosomal genes are the plasmids. Plasmids are small, circular molecules of DNA that are capable of replicating

independently. *S. aureus* strains carry at least one or more plasmids ranging in size from 1 to 60kb. The plasmids are classified into three main classes based on size and ability to conjugate.

Class I consist of small plasmids (1-5kb) occurring between 10 to 55 copies per cell [85]. The plasmids are either cryptic or carry a single or in rare instances two resistance determinants. Four families made up class I namely pT181, pC194, pSN2 and pE194. The pT181 family contain plasmids conferring chloramphenicol, tetracycline and streptomycin resistance. pC194 family contains plasmids conferring resistance to chloramphenicol, aminoglycosides, quaternary ammonium compounds and cadmium. Both pSN2 and pE194 plasmid family confers resistance to macrolide, lincosamides and streptogramin B [83]. Class II plasmids (15-30kb) are relatively larger in size compared to class 1 and exist between 4 to 6 copies in a cell. The resistance genes are often associated with transposons that have integrated into the plasmid genome. They are mainly associated with resistance to penicillin, aminoglycosides or trimethoprim resistance [86]. Class III plasmids (30-60k) consists of even larger plasmids and carry a number of different resistance determinants for aminoglycosides, trimethoprim, quaternary ammonium compounds and some beta lactams [87].

In addition to plasmids providing a relatively efficient means for staphylococci to exchange genetic information such as antibiotic resistance genes and virulence determinants, a comprehensive knowledge of plasmid content is also critical for a full understanding of pathogen diversity [88]. Complete genomic analysis of plasmids in strains of *S. aureus* clone USA300 revealed high level of identity among recent isolates but relatively divergent from earlier USA300 isolates that comprise the epidemic clone. The plasmid diversity is probably due to varied selective pressures imparted to the parent strain through acquisition of plasmid-

mediated antibiotic resistance which is common in health care-associated isolates than in those from the community [89].

1.11.6 *S. aureus* sequence types

The unambiguous characterization of bacterial isolates in a standardized, reproducible, and portable manner was first proposed in 1998 using multilocus sequence typing (MLST) approach [90]. MLST has been successfully used to differentiate and trace *S. aureus* species that cause outbreaks or epidemics of infectious diseases. In the process, the sequences of internal fragments of several housekeeping genes are determined for each isolate, thereby defining specific alleles for each locus. In *S. aureus*, seven housekeeping genes (loci) (arcC, aroE, glpF, gmk, pta, tpi, and yqiL) provides a unique allelic profile known as sequence types (STs) [91] **Figure 5**. As there are many alleles at each of the seven loci, isolates are highly unlikely to have identical allelic profiles by chance, and isolates with the same allelic profile can be assigned as members of the same clone [92, 93]. So far, more than 6000 *S. aureus* STs have been reported in the sequence/profile definitions database of the MLST website https://pubmlst.org/bigsdb?db=pubmlst_saureus_seqdef&page=downloadProfiles&scheme_id=1

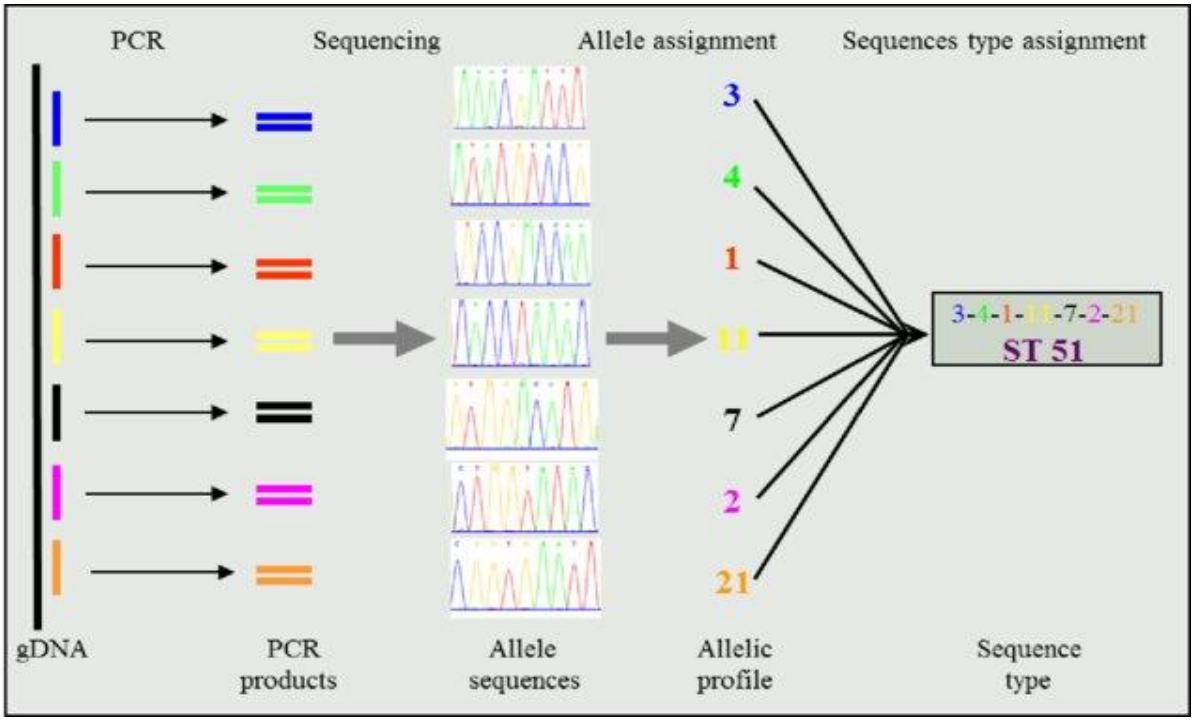


Figure 5 Scheme for multilocus sequence typing reproduced from https://www.researchgate.net/profile/Werner_Ruppitsch/publication/313256513/figure/fig6/AS:457794219450371@1486158055299/Scheme-for-multilocus-sequence-typing-adapted-from-mlstnet-MLST-uses-sequence_W640.jpg. The scheme uses sequence variations in up to seven housekeeping genes. Allele numbers are assigned to unique sequences and the allele number combination result in a ST.

Groups of STs in which every ST shares at least five of seven identical alleles with at least one other ST in the group are referred to as clonal complexes [94]. The following clonal complexes: CC1, CC5, CC8, CC15, CC22, CC30, CC45, CC97 and CC93 are most frequently seen in the MLST database. They can be assigned unambiguously, with only minimal overlap with other complexes.

Among 26 European countries between September 2006 and February 2007, 2,890 *S. aureus* isolates were collected from patients with invasive *S. aureus* infection. The six most common methicillin sensitive *S. aureus* (MSSA) and methicillin resistant *S. aureus* (MSRA) were STs 5, 15, 45, 7, 30, 1 and STs 22, 8, 111, 5, 228, 5 respectively [95]. In the USA, MLST was determined for *S. aureus* bacteraemia cases in 5 academic medical centres between 2008 and 2011. The 3 most common MSSA STs were ST5, ST8, and ST30 whilst ST8 and ST5 accounted for 83% of the MRSA isolates [96]. In Africa, the six most common MSSA and MSRA sequence types were reported to be ST5, ST8, ST15, ST30, ST121 , ST125 and ST5, ST8, ST80, ST88, ST239/241 respectively [12]. *S. aureus* STs appear to vary from one country to another. Among children aged less than 15 years in the Ashanti region of Ghana, the dominant sequence type was reported to be ST152 followed by ST15 and then ST45 [50]. The STs rarely carry the *mecA* gene therefore rates of MRSA were low. However, prevalence of Panton-Valentine leucocidin (PVL) is high hence the STs has the potential to cause severe invasive infections [50] as PVL is a virulence factor that targets white blood cells causing leukocyte destruction and tissue necrosis [97]. A similar prevalence of sequence types was also observed among Ghanaian healthcare institutions [98] suggesting that the carriage STs were responsible for infections. In Kenya, the dominant *S. aureus* sequence types from selected isolates from private and public referral hospitals were ST1, ST8 and ST241 [99]. These STs were mostly MRSA and also resistant to a number of antibiotics including clindamycin, erythromycin and trimethoprim/sulfamethoxazole. *S. aureus* isolated from academic centres in South Africa revealed ST612 as the dominant ST [100] whereas clonal complex 5 (CC5) was the most dominant among patients in the surgical ward in Nigerian university hospital [101]. In both countries, the dominant ST were methicillin resistant with the South African ST mainly hospital acquired.

In The Gambia, there is limited data on the prevalence of *S. aureus* sequence types. Ebruke C. *et al.* [102] reported 59 different sequence types of *S. aureus* isolated from nasopharyngeal samples from all age groups of which the dominant sequence types were ST15(28%) and ST5(4%). Like in carriage, the dominant *S. aureus* sequence types from clinical isolates were ST15 and ST5 [103].

1.11.7 Antibiotic treatment options for *S. aureus* infections in The Gambia

The Gambia national standard treatment guideline is the recommended guide for all clinical staff in the treatment of *S. aureus* infection in the Gambia. However, there is no strict monitoring for adherence by The Gambia medical and dental council or the ministry of health. Whilst some follow the national guidelines, others use the World Health Organisation guidelines and the rest especially those in the rural areas of the country are forced to treat suspected *S. aureus* infections with the available antibiotics. Overwhelmingly, in hospital settings where microbiology laboratory exists, laboratory confirmed *S. aureus* infection is treated with cloxacillin as the first line [11]. The second line treatment options include Amoxiclav or Augmentin and macrolides such as azithromycin. The last line of treatment is vancomycin which is rarely available to the clinical staff.

Data on the antibiotic susceptibility pattern for *S. aureus* isolates from invasive diseases and skin and soft tissue infections (SSTIs) for the cloxacillin, amoxiclav, macrolides or vancomycin are limited. Between 2003 and 2005, 871 patients admitted to Medical Research Council, The Gambia ward suspected for bacteraemia had blood culture taken and 18.3% of the samples were positive for *S. aureus*. All isolates were susceptible to cloxacillin, gentamicin and chloramphenicol; moderately susceptible to co-trimoxazole (66.7%) and poorly susceptible to

tetracycline (33%) and penicillin (8%) [30]. In a recent publication, 293 *S. aureus* isolates from a cross sectional study based on data compiled from records and samples for patients seen at The MRCG clinic during the period January 2005—December 2015 were screened for antibiotic susceptibility. Prevalence of antimicrobial resistance was low and included chloramphenicol (4.8%), cefoxitin (2.4%), ciprofloxacin (3.8%), erythromycin (8.9%), gentamicin (5.5%) penicillin (92.5%), tetracycline (41.0%), and sulfamethoxazole-trimethoprim (24.2%) [104].

1.11.8 *S. aureus* macrolide resistance genes

Three main mechanisms of *S. aureus* macrolide resistance encoded by genes have been described. Firstly, through target-site modification by methylation or mutation that prevents the binding of the antibiotic to its ribosomal target. Secondly, through efflux of the antibiotic, and finally by drug inactivation [105]. **Figure 6** shows the three mechanisms of macrolide resistance

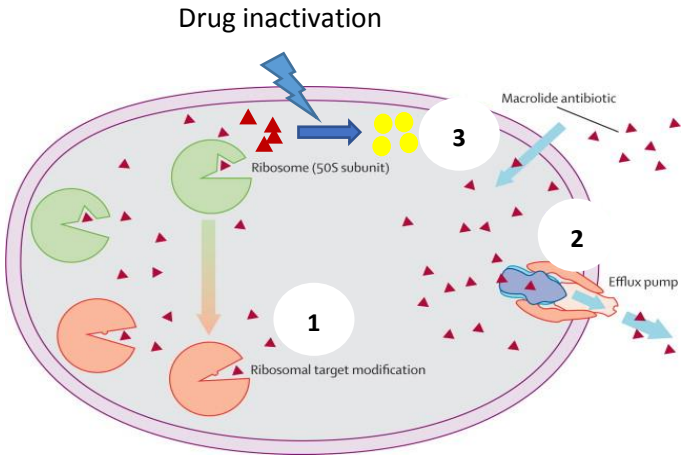


Figure 6. The 3 main mechanisms of macrolide resistance coded for my by the *erm, msr* and *mph/ere* genes adapted from [106]. 1=target modification, 2= efflux pump and 3= drug inactivation

In the first mechanism, an adenine nucleotide at position 2058 in the 23S rRNA subunit which plays a key role in the binding of macrolides and other antibiotics such as lincosamides and streptogramin B may be methylated by an enzyme encoded by erythromycin ribosome methylase (*erm*) gene. This methylation results in the impairing of the binding of macrolides, lincosamides and streptogramin B (MLSB) antibiotics to its target leading to resistance. In pathogenic bacteria, nearly 40 *erm* genes have been reported [107] and are mostly borne by plasmids and transposons. These *erm* genes have now been grouped based on their amino acid sequence similarity into 21 classes; 4 of which are present in pathogenic bacteria. The four major classes are: *erm*(A), *erm*(B), *erm*(C), and *erm*(F) [108]. The *erm*(A) and *erm*(C) genes are typically found in staphylococci [105]. Expression of macrolides lincosamides and streptogramin B(MLSB) resistance can be either constitutive or inducible. In inducible resistance, the bacteria produce inactive mRNA that is unable to encode methylase. The mRNA becomes active only in the presence of a macrolide inducer such as 14 or 15 membered lactone ring macrolides. By contrast, in constitutive expression, active methylase mRNA is produced in the absence of an inducer [105].

The second mechanism of macrolide resistance in staphylococci is through the expression of efflux pumps. These pumps are ABC transporters encoded by plasmid borne genes called macrolide and streptogramin resistance genes msr(A/B). Unlike chromosomal encoded pumps in Gram-negative bacteria that contribute to intrinsic resistance to hydrophobic compounds [109], these efflux pumps are specific to macrolides and streptogramin B antibiotics [110].

Finally, resistance could be due to inactivation of the macrolide by two enzymes namely phosphotransferases and esterases encoded by *mph*C and *ere*A/B genes respectively.

phosphotransferases inactivates macrolides by phosphorylating a hydroxyl group on the macrolide whereas esterases hydrolysis the lactone ring [109].

There are currently very few data in The Gambia on the prevalence of genes associated with macrolide resistance following azithromycin interventions. A single study described prevalence of antibiotic resistance determinants following mass drug administration of azithromycin from 23 *S. aureus* isolates by phenotype. The results showed that no macrolide resistant determinants were found in six isolates that were sensitive to both azithromycin and clindamycin. Five isolates displaying resistance to azithromycin but sensitivity to clindamycin were positive for the *msr* gene, which conveys resistance to macrolides and streptogramin B. Twelve isolates were azithromycin resistant and had the iMLSB phenotype and all of these carried *erm* genes confirming their resistance to the MLSB antibiotics [111].

1.11.9 Transmission of *S. aureus* macrolide resistant genes

S. aureus macrolide resistant genes are predominantly located on plasmids that are easily transmissible. *S. aureus* like many bacteria has the ability to integrate genetic material coming from more or less evolutionarily distant organisms. This mechanism is called “horizontal gene transfer” as opposed to vertical transmission from mother to daughter cell [112]. Three mechanisms have been described namely transformation, conjugation and transduction **Figure 7**.

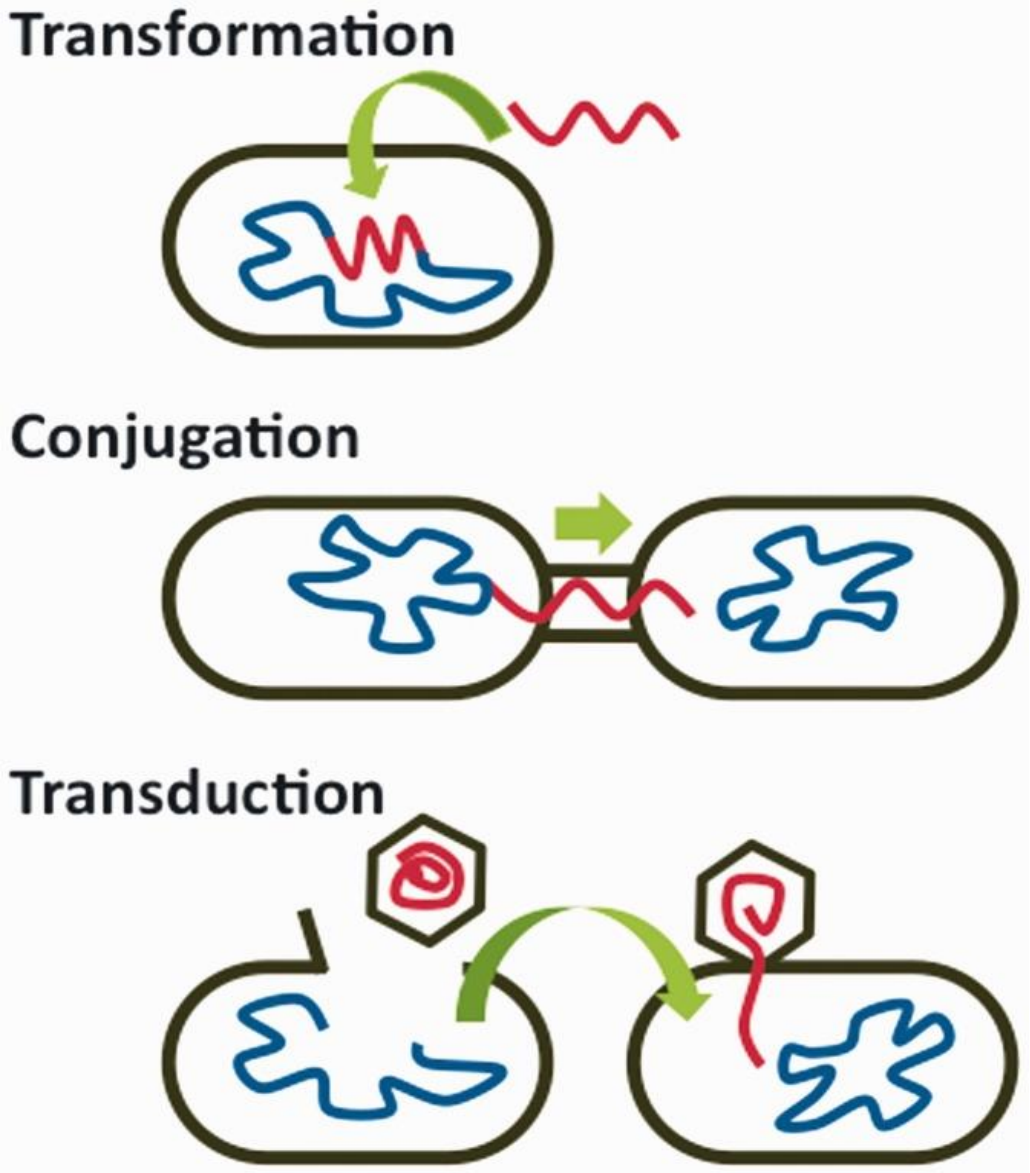


Figure 7. The three mechanisms of horizontal gene transfer between bacteria reproduced from [112] namely transformation, conjugation and transduction.

Transformation simply refers to bacteria taken up free DNA from dead organisms into the cytoplasm. Conjugation on the other hand refers to transfer of genetic material to another through direct contact via sexual pilus whilst transduction is a type of transfer that occurs via a bacteriophage that transmits the DNA from one cell to another [113].

Transfer of macrolide resistant gene between bacteria colonising an individual could result in the emergence of phenotypically resistant bacteria strains that may eventually spread in within the population. These bacteria may be resistant to a number of antibiotics making them very difficult to treat.

1.12. Other *Staphylococci species*

1.12.1 General characteristics

The genus staphylococcus includes at least 50 different species [114]. Majority of these *Staphylococci species* are harmless and reside normally on the skin and mucous membranes of humans and other organisms [115]. They may also be isolated from food products where they may be involved in fermentation or from other animals. *Staphylococcus spp.* have also been reported to cause a wide variety of diseases in humans. *S. saprophyticus, S. epidermidis, S. lugdunensis, and S. schleiferi* have been implicated in a number of human infections [115-117]. In addition, some coagulase-negative staphylococci (CoNS) have also emerged as important pathogens in hospital-acquired infections [115]. They are particularly associated with the use of indwelling or implanted foreign bodies and often transmit from one patient to another through medical and or nursing procedures [118].

1.12.2 Staphylococci and macrolide resistance genes

Staphylococci species other than *S. aureus* are also known to carry genes associated with macrolide resistance. The *msr*A gene that codes for an efflux pumps which actively pumps

macrolides and streptogramins out of the bacterial cell was first identified in *Staphylococcus epidermidis* [110]. There are currently no data on the prevalence of macrolide resistance genes among other *Staphylococcal species* in the Gambia. However, in Turkey, prevalence of macrolide resistant genes among erythromycin-resistant clinical isolates of coagulase-negative staphylococci was reported to be 30% for *erm*C and 32% for *msr(*A) [119]. In a study conducted in Germany among erythromycin resistant coagulase negative staphylococci, most (63%) erythromycin-resistant isolates carried constitutively expressed *ermC* as the sole resistance determinant and the *msr(A)* gene was carried by 20–30% of all erythromycin-resistant isolates [120]. Macrolide resistance genes were also described among *S. saprophyticus* in France [121], *Staphylococcus species* in Argentina [122] and *S. epidermidis* from blood stream infection in Belgium [123].

Coagulase-negative staphylococci (CoNS), particularly *Staphylococcus epidermidis*, can be regarded as potential reservoirs of resistance genes for pathogenic strains [124]. Since these genes are usually located on plasmids and *Staphylococci species* including *S. aureus* co-colonise an ecological niche at the same time, the genes can easily transfer between same or different bacterial species through transformation, conjugation or transduction [112].

1.13 Azithromycin interventions on *S. aureus* carriage and resistance

Azithromycin is a macrolide antibiotic belonging to the azalide subclass [125]. Structurally, azithromycin is related to erythromycin but has a broader spectrum of antibacterial activity, improved tissue penetration and a more favourable pharmacokinetic profile [126].

Azithromycin prevents bacteria from growing by interfering with their protein synthesis. It binds to the 50S subunit of the bacterial ribosome and thus inhibits translation of mRNA [127].

Azithromycin has been used in mass drug administration (MDA) campaigns to control trachoma in different countries in Africa [128-131]. Such campaigns have beneficial effects beyond the target disease. In a study in Ethiopia, MDA with azithromycin for trachoma control reduced all-cause mortality in 1-9 years old children by 49% [132] and in The Gambia MDA reduced asymptomatic pneumococcal carriage by 24.2% in a rural population within a space of just one month [133]. Azithromycin has also been used during pregnancy. Randomized clinical trials conducted in Malawi and Papua New Guinea have shown that azithromycin may help to prevent low birth weight when given in combination with sulphadoxine pyremithamine during the second and third trimester of pregnancy [134, 135].

Azithromycin interventions have been shown in a number of clinical trials and MDA to significantly reduce carriage of *S. aureus* but also associated with increased prevalence of azithromycin resistance *S. aureus*. There does not appear to be much data on the impact of azithromycin interventions and emergence of macrolide resistant *S. aureus* in the African sub region. However, in Papua New Guinea, the proportion of azithromycin resistant *S. aureus* isolates was significantly higher among pregnant women receiving azithromycin as part of intermittent preventive treatment (79.6% [78/98], versus 14.4% [15/104]; $P < 0.001$) [134]. In the same region, children living in rural Australia or urban New Zealand with bronchiectasis on long term azithromycin treatment for 24 months reports 100% macrolide resistance among *S. aureus* isolates [136]. Among cystic fibrosis patients in Denmark, continuous treatment with azithromycin for at least 3 months reduced prevalence of carriage of *S. aureus* but increased macrolide resistant *S. aureus* (7% to 52.5% $p<0.001$) [137].

In The Gambia, three annual rounds of azithromycin MDA was associated with a short-term increase in both prevalence of carriage of azithromycin and inducible resistance to macrolide lincosamides and streptogramin B (MLS_B) resistant *S. aureus* [111].

1.14 The PregnAnZI 1 trial

1.14.1 Trial design and objectives

The PregnAnZI 1 trial was a Phase III, double -blind, placebo controlled randomized clinical trial. As part of this trial, pregnant women in labour attending study health facilities were randomized to receive either a single dose of 2 g of oral azithromycin or placebo (ratio 1:1). The primary objective of the trial was aimed at reducing bacterial carriage in both mother and new-born as bacterial carriage is a necessary step for infection leading to disease hence reducing carriage may help to reduce disease.

1.14.2 Study setting

The PregnAnZI 1 trial was conducted in Bundung, a peri-urban setting located in the western region of The Gambia **Figure 8**.

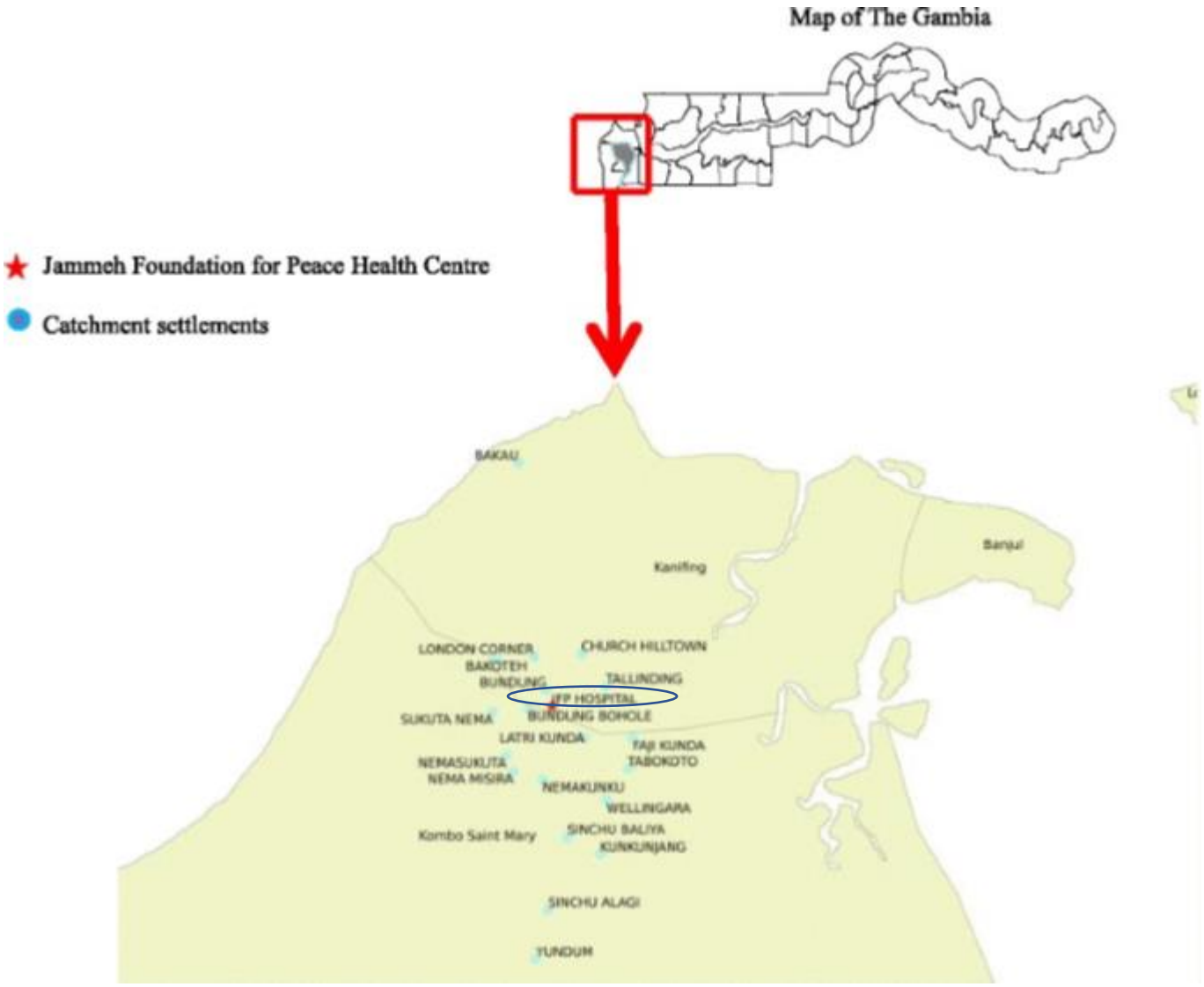


Figure 8. The figure shows the map the Gambia and the study site (Bundung maternal and child health hospital formerly called Jammeh Foundation for Peace Health Centre) with the surrounding catchment settlements.

It is a very densely populated area with majority of the population engaged in a wide range of occupations, including working for government services, liberal professions and trading. The climate of the area is typical of the sub-Sahel region, with a long dry season from

November to May and a short rainy season between June and October. The main hospital called the Bundung Maternal and child health hospital (BMCHH) formerly referred to as the Jammeh Foundation for Peace Hospital (JFPH), is approximately 8km from Fajara where the main MRC laboratories are located. The hospital serves a local community of more than 50,000 inhabitants and manages approximately 5, 000 deliveries per year.

1.14.3 Screening, recruitment and randomization

Pregnant women attending the JFPH for antenatal care and planning to deliver their babies there were introduced to the study. Interested women were invited to the study office for a detailed explanation in the presence of an impartial witness. Those that gave consent had unique sensitization sticker placed on their antenatal card indicating agreement to join the study. When one of the study pregnant women in labour visits the labour ward of the hospital, the study nurse confirms she gave consent and also assess her willingness to continue participating in the study. Women aged 18 to 45 years were included except any of the following exclusion criteria were found:

Known HIV infection, any chronic or acute condition that might interfere with the study as judged by the research clinician, planned travel out of the catchment area during the 2 months after delivery (follow-up period), planned caesarean section, known required referral, known multiple pregnancy, known severe congenital malformation of the baby, intrauterine death confirmed before randomization, known allergy to macrolides, intake of antibiotics in the week before randomization.

The woman was recruited into the study once eligibility was determined. This was followed by collection of baseline or pre-intervention samples from the mother [nasopharyngeal swab (NPS) and vaginal swab].

Azithromycin and placebo were provided as tablets packed in blisters labelled from 001 to 830. Each woman received 2g of azithromycin or placebo. The time when the drug was taken was recorded. The randomization number on the specific blister was written in the spaces provided on the particular page of the case report form (CRF).

1.14.4 Ethical approvals

All study mothers had signed consent during their pre-natal visits before being enrolled into the trial. A local safety monitor (LSM) and a Data Safety Monitoring Board (DSMB) reviewed all the serious adverse events (SAE) during the course of the trial. The trial was monitored by an independent clinical trials monitor. Approval of the study was by the joint Medical Research Council at London School of Hygiene & Tropical Medicine /Gambia Government Ethics Committee.

1.14.5 Sample collection and storage

Following delivery, women and new-born pairs were then followed for 8 weeks collecting nasopharyngeal swabs (NPS), breast milk (BM) and vaginal swabs (VS) during the first 4 weeks of the follow-up (**Table 1**). Following collection, the samples were transported to the laboratory within 8hrs and stored at -70°C.

Table 1. The different samples types (NPS, vaginal swab and breast milk,) collected from mothers and new-borns during a 28 day the follow-up period.

	Day 0 Hospital	Day 3 Home visit	Day 6 Home visit	Week 1 Day 8-10 Hospital	Week 2 Day 14 Home visit	Week 4 Day 28 Home visit
Mother						
Vaginal swab	**X**			**X**		
NPS	**X**	**X**	**X**		**X**	**X**
Breast milk		**X**	**X**		**X**	**X**
New-born						
NPS	**X**	**X**	**X**		**X**	**X**

1.14.6 Bacteriological analysis for NPS, VS and BM samples

Frozen NPS, BM or VS were allowed to thaw on ice. Each vial was then vortexed briefly in order to homogenise the medium and 50 µl was dispensed onto gentamicin blood agar (GBA) (CM0331 Oxoid, UK +5 % sheep blood), mannitol salt agar (MSA)(CM0085 Oxoid, UK) and crystal violet blood agar (CVBA)(CM0085 Oxoid, UK +0.02 % crystal violet) for selective isolation of *S. pneumoniae*, *S. aureus* and group B streptococci (GBS) respectively. Alpha haemolytic colonies from GBA plates that were optochin sensitive and or bile soluble were

confirmed *S. pneumoniae*. Pale to golden yellow domed shaped colonies from MSA plates that were catalase and Staphaurex™ Plus Latex Agglutination test positives were confirmed to be *S. aureus*. Presumptive beta-haemolytic colonies from CVBA plates that were catalase negative were confirmed to be streptococci and grouped using the Streptex grouping kit (Remel R30950501) and ultimately reported as A, B, C, D, F or G.

1.14.7 Antibiotic susceptibility testing

For each confirmed bacteria, a 0.5 % McFarland's Standard was made in normal saline. A sterile swab was then immersed into the suspension and then streaked evenly over the surface of the Mueller Hinton agar (MHA) with 5 % sheep blood (*S. pneumoniae* and GBS) and MHA without blood (*S. aureus*) in three directions, rotating the plate 90° to ensure even distribution. Azithromycin resistance was screened by disk diffusion (15 µg azithromycin disc) and those intermediate or resistant were determined by E-test following the clinical and laboratory standard institute (CLSI) 2014 guidelines for the performance of test and interpretation of results: (i) *S. aureus* isolates with E-test values ≥8 µg/ml were reported as resistant whilst values <8 µg/ml ≥ 4 µg/ml as intermediate resistant; (ii) *S. pneumoniae*/GBS isolates with E-test values ≥2 µg/ml were reported as resistant whilst values <2 µg/ml ≥ 1 µg/ml as intermediate resistant.

1.14.8 PregnAnZI 12 months cross-sectional

Between November 2014 and May 2015, children aged 12 months (range 11-13 months) whose mothers had participated in the PregnAnZI 1 trial were visited at home and their parents re-consented by a trained nurse blinded to the treatment allocation. NPS were collected using calcium alginate (Expotech USA Inc.) swab as described previously [138]. In brief, each swab was immediately placed into a vial containing skim milk-tryptone-glucose-glycerol (STGG) transport medium and then into a cold box before being transported to the MRC Laboratories within 8 hours [139]. Upon receipt in the laboratory, the tubes were vortexed for a minimum of 20 seconds before being stored in freezers at -70ºC.

1.14.9 Results

Prevalence of nasopharyngeal carriage of *S. aureus*, GBS or *S. pneumoniae* in the new-borns at day 6 was lower in the intervention arm (28.3% versus 65.1% prevalence ratio 0.43; 95% CI 0.36–0.52, p <0.001). At the same time-point, prevalence of any of the above three bacteria in the mother was also lower in the azithromycin group (nasopharynx, 9.3% versus 40.0%, p <0.001; breast milk, 7.9% versus 21.6%, p <0.001; and the vaginal tract, 13.2% versus 24.2%, p <0.001) [56].

Similarly, prevalence of *S. aureus* carriage was also less common among women (i.e. NPS, VS and BM samples) and in new-borns (i.e. NPS) from the azithromycin arm during at least 4 weeks following the intervention. At day 6 (primary endpoint of the trial), the prevalence of nasopharyngeal carriage of *S. aureus* in new-borns was 27.5% in the azithromycin arm versus 63.3% in the placebo arm [prevalence ratio (PR)=0.43 95%CI 0.36-0.52] **Figure 9a** [56]. Among mothers, *S. aureus* nasopharyngeal carriage was also lower in the azithromycin arm during

the 4 weeks following the intervention (at day 6, 7.8% compared to 27.2% in the placebo arm PR=0.29 95%CI (0.20-0.42)] **Figure 9b**. The same observations were made between arms for breast milk or vaginal samples **Figure 9c** and **Figure 9d** [56].

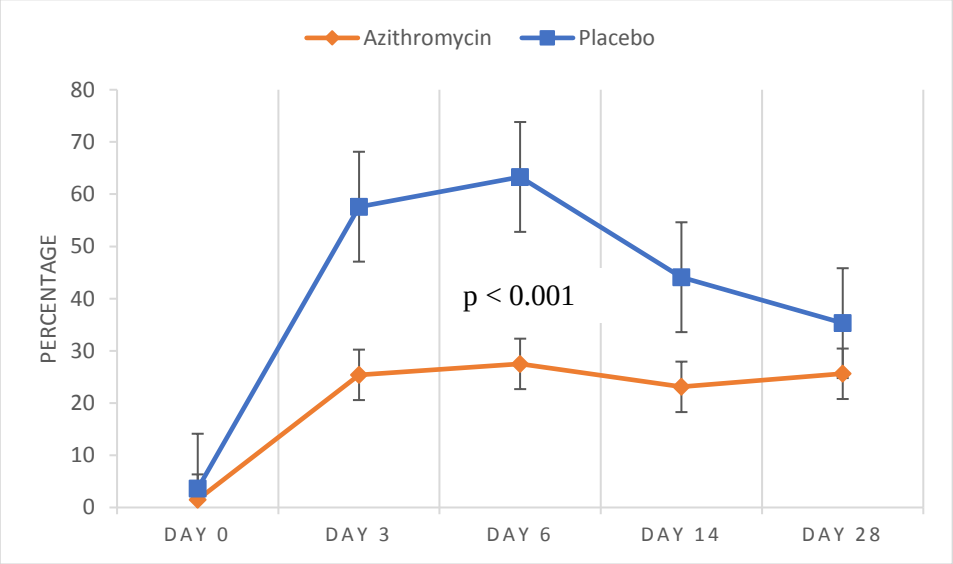


Figure 9a. Prevalence of carriage of *S. aureus* during follow up period in new-borns NPS among all new-borns. Carriage was similar at day 0, decreased significantly in the intervention arm during the follow up period

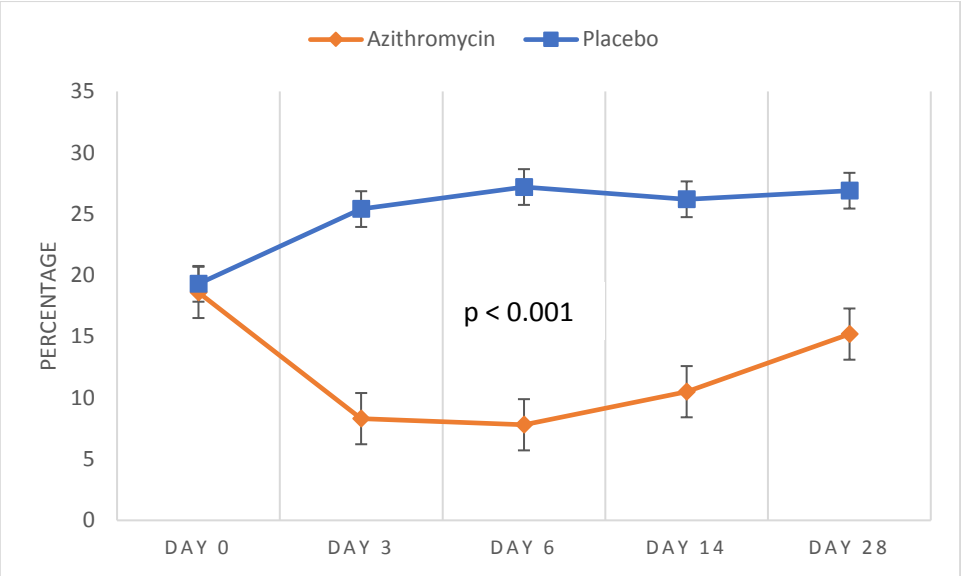


Figure 9b. Prevalence of carriage of *S. aureus* during follow up period in mothers NPS among all study mothers. Carriage was similar at day 0, decreased significantly in the intervention arm during the follow up period

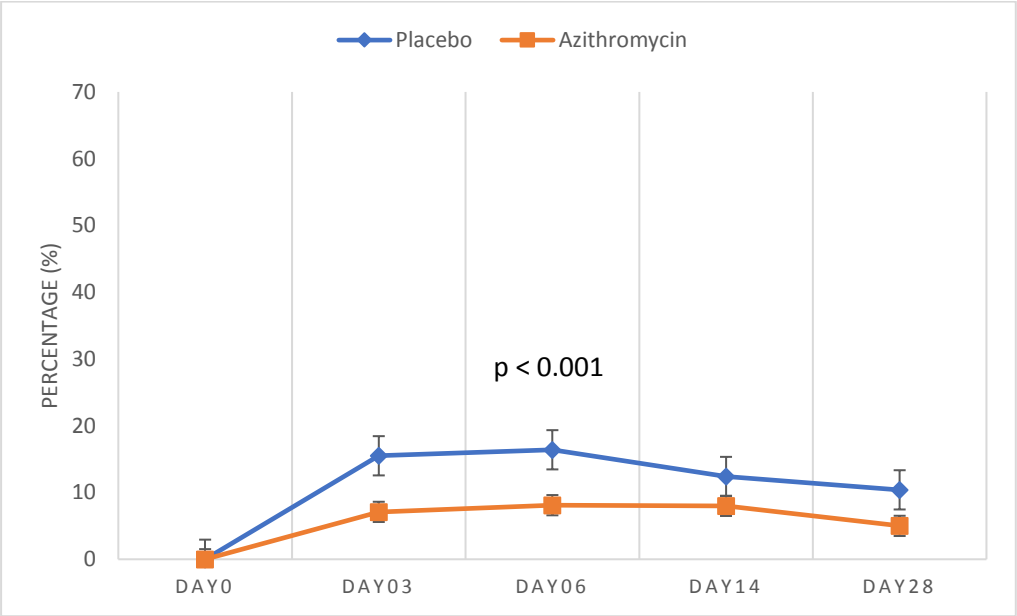


Figure 9c. Prevalence of carriage of *S. aureus* during follow up period in mothers breast milk among all study mothers. Carriage was similar at day 0, decreased significantly in the intervention arm during the follow up period

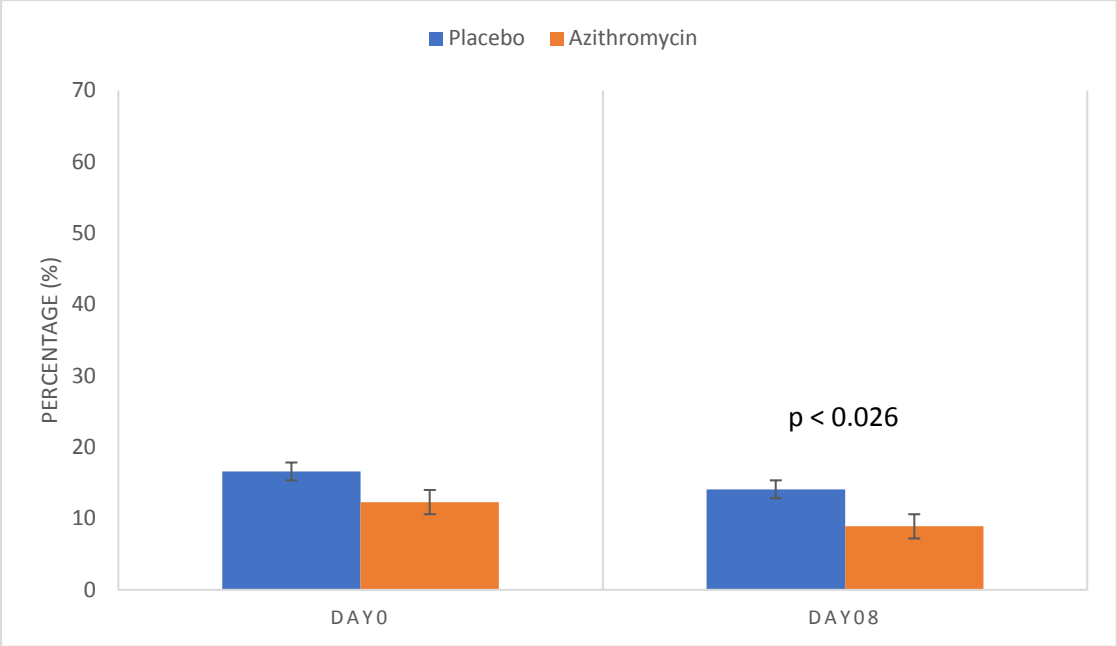


Figure 9d. Prevalence of carriage of *S. aureus* during follow up period in mothers' vaginal track among all study mothers. Carriage was lower both at day 0 and day 8 in the intervention.

On the other hand, there was a significant increase in the prevalence of azithromycin resistant *S. aureus* in the nasopharynx of neonates; from 0.7% at day 0 to 16.7% at day 28 **Fig 10a**. Among mothers, nasopharyngeal prevalence of azithromycin resistant *S. aureus* carriage was also higher in the azithromycin arm with 1.4% at day 0 to 12.6% at day 28 **Fig 10b**. Observations made between arms for breast milk or vaginal samples followed the same pattern as above **Fig 10c** and **Fig 10d** respectively [56].

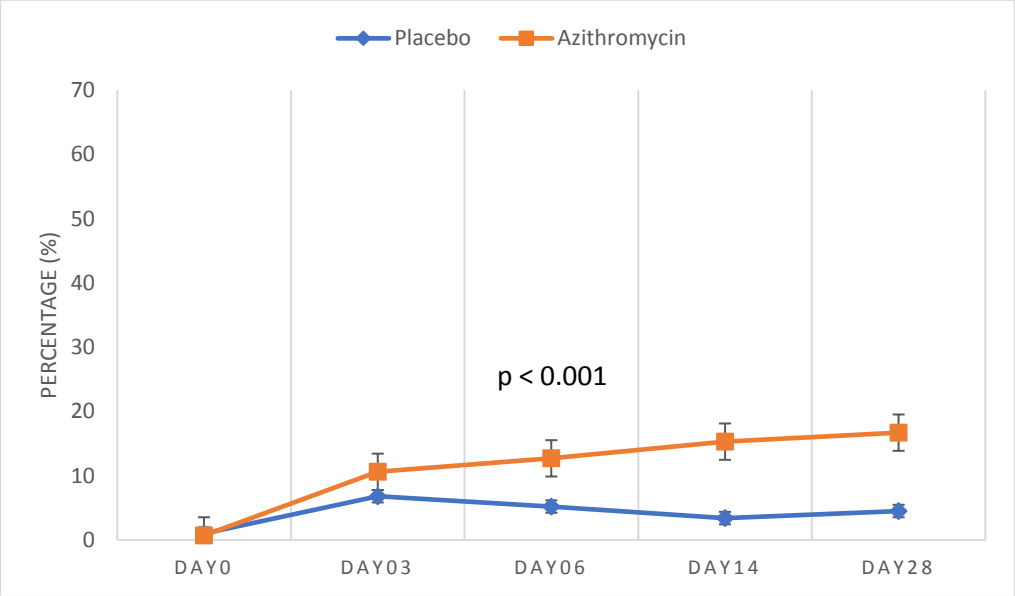


Figure 10a Prevalence of azithromycin resistant *S. aureus* carriage during follow up in new-borns' NPS among all new-borns. Carriage was similar at day 0 and increased in the intervention arm during the follow up period.

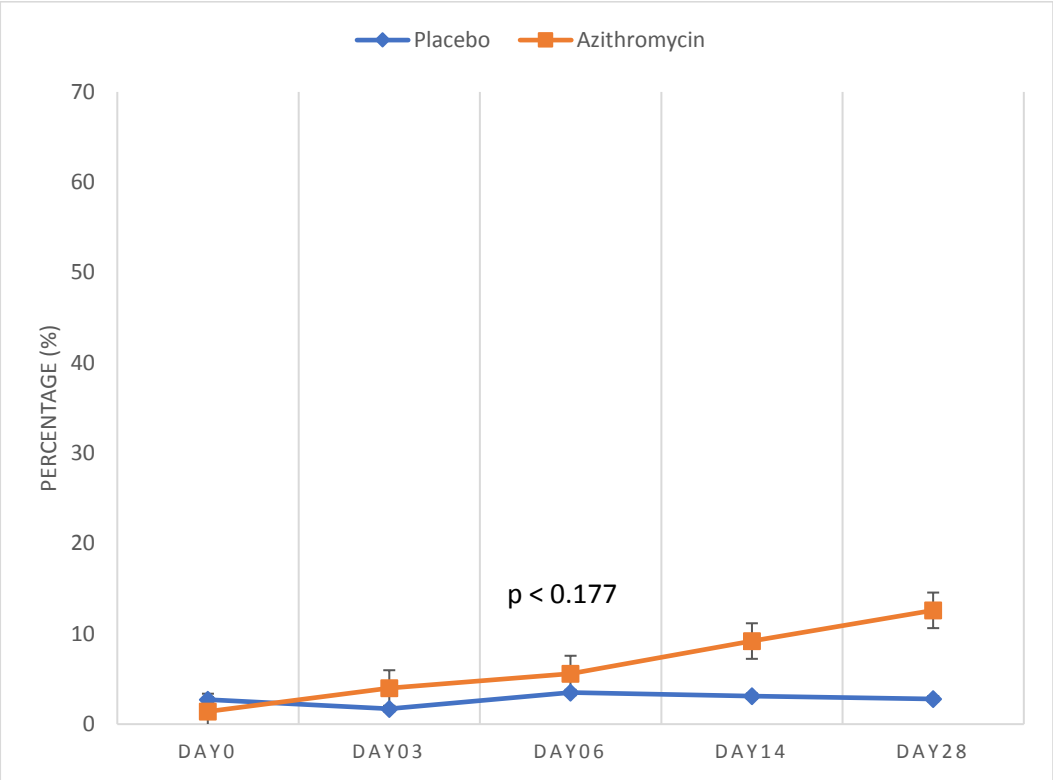


Figure 10b Prevalence of azithromycin resistant *S. aureus* carriage during follow up in mothers' NPS among all study mothers. Carriage was similar at day 0 and increased in the intervention arm during the follow up period.

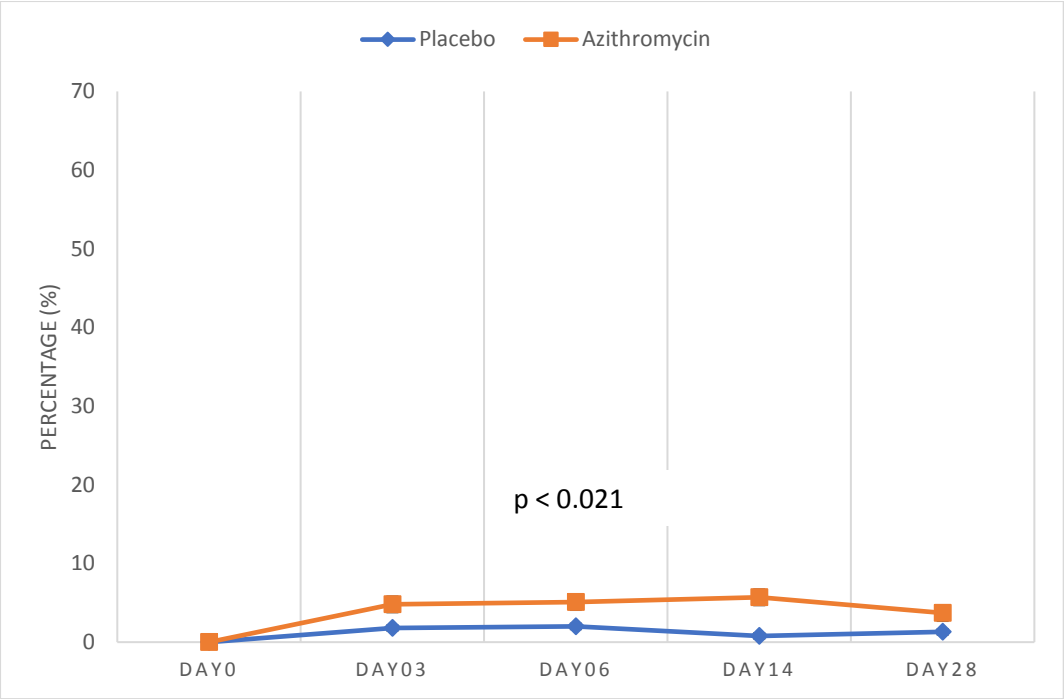


Figure 10c Prevalence of azithromycin resistant *S. aureus* carriage during follow up in mothers' breast milk among all study mothers. Carriage was similar at day 0 and increased in the intervention arm during the follow up period.

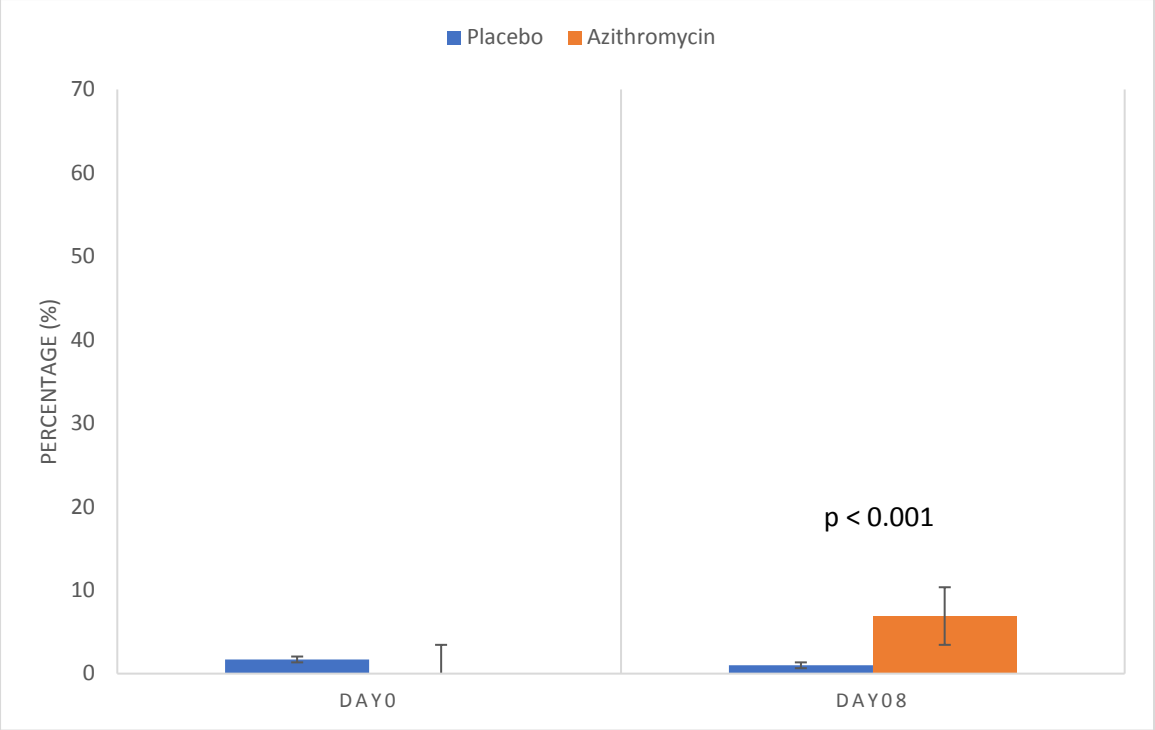


Figure 10d Prevalence of azithromycin resistant *S. aureus* carriage during follow up in mothers' vaginal track among all study mothers. By day 8 almost all isolates were resistant in the intervention arm.

In a post hoc analysis, it was observed that the trial showed a significant reduction in clinical conditions such as mastitis, puerperal sepsis, related infections among mothers and any kind of infection among new-borns in the azithromycin arm compared to those in the placebo arm **Fig 11** [140].

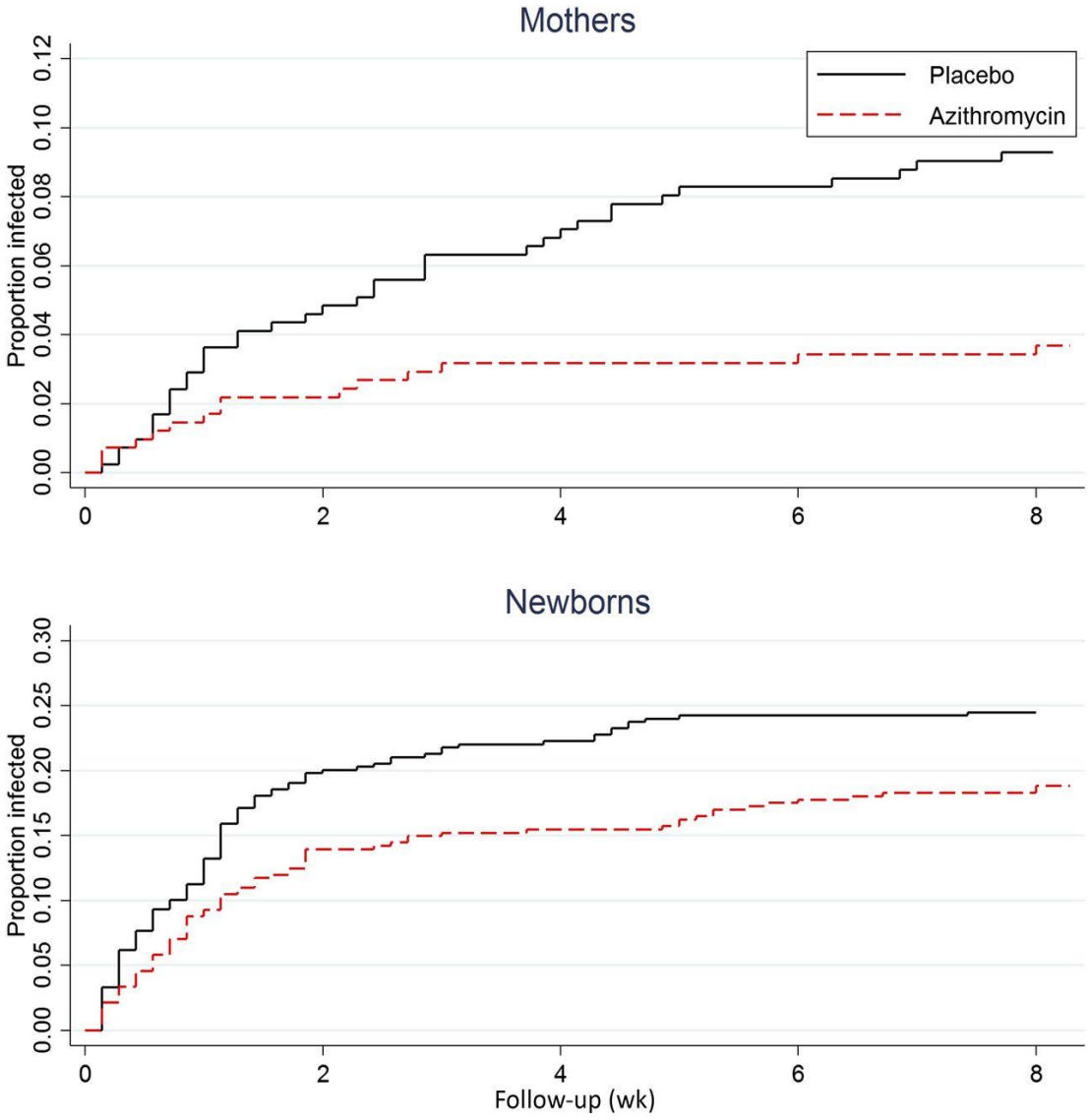


Figure 11. Infection rate among mothers (mastitis and puerperal sepsis) and new-borns (skin and any kind of infection) in the azithromycin or placebo arms of the PregnAnZI trial during 8 weeks of follow up reproduce from [140]. Rates were lower for both mothers and new-borns in the intervention arm

Incidence of purulent conjunctivitis due to bacterial infection was also lower in the azithromycin arm 1.2% vs. 3.8% OR 0.31 95%CI (0.12-0.82) and *S. aureus* being the most common bacterial species associated with these infections [112] **Table 2.**

Table 2 Incidence of bacterial conjunctivitis reported by trial arm

Characteristic	**Azithromycin arm** (*N* = 419) *n* (%)	**Placebo arm** (*N* = 424) *n* (%)	**OR** (95% CI)	***p*-value**
Purulent discharge	18 (4.3)	22 (5.2)	0.82 (0.44,1.54)	0.628
Any bacteria detected	5 (1.2)	16 (3.8)	0.31 (0.12,0.82)	0.025
Any gram-positive	4 (1.0)	14 (3.3)	0.28 (0.10,0.82)	0.029
S. aureus	4 (1.0)	12 (2.8)	0.33 (0.11,0.98)	0.074
S. pneumoniae	0 (0.0)	2 (0.5)	0.00 (0.00,1.94)	0.499
Any gram-negative	1 (0.2)	6 (1.4)	0.17 (0.00,1.06)	0.123
H. influenzae	0 (0.0)	3 (0.7)	0.00 (0.00,1.29)	0.249
M. catarrhalis	0 (0.0)	3 (0.7)	0.00 (0.00,1.29)	0.249
N. gonorrhoeae	1 (0.2)	1 (0.2)	1.01 (0.00, NA)	1.00
C. trachomatis	0 (0.0)	0 (0.0)	–	–
Bacterial co-infection[a]	0 (0.0)	4 (0.9)	0.00 (0.00,0.97)	0.124

[a]Bacterial co-infections: (i) *S. aureus* and *H. influenzae*; (ii) *S. pneumoniae* and *H. influenzae*; (iii) *S. aureus* and *N. gonohorreae* (iv) *M. catarrhalis*, *S. pneumoniae* and *H. influenzae*

1.14.10 Summary of results

As a summary, 2g of azithromycin given to women in labour had a strong effect on prevalence of *S. aureus* carriage in mothers and their babies and had an effect on non-severe neonatal and maternal infections. Although the intervention has a potential important public health impact, on reducing maternal and neonatal infections, the effect of the intervention on long term carriage of pathogenic bacteria and antibiotic resistance, especially for *S. aureus*, needs serious attention. This will be the focus of my PhD and the investigations undertaken in order to examine those effects will be outlined in subsequent chapters of this thesis.

1.15 PhD Study rationale

The PregnAnZI 1 trial aimed at lowering bacterial carriage both in the mother and her new-born, a necessary step to reducing maternal and neonatal sepsis. Results of the trial revealed that intrapartum azithromycin decreased carriage of *S. aureus*, group B *Streptococcus* and *Streptococcus pneumoniae* during the entire neonatal period for both the women and the new-borns. However, it also resulted to increased prevalence of azithromycin resistant *S. aureus* during the 28-day follow-up period from both mothers and new-borns.

A prophylactic intervention such as intrapartum oral azithromycin may eventually be implemented only after carefully balancing the benefits versus the risks. One of the major unintended consequences, or risks, of antibiotic prophylaxis is the emergence of antibiotic resistant bacterial species which can potentially spread within the community leading to infections that become more difficult to treat.

Among the three bacteria tested as part of the PregnAnZI 1 trial, azithromycin resistance to *S. aureus* increased among mothers and babies exposed to azithromycin and hence warranted further investigation.

CHAPTER 2

2.1 Aims

Intrapartum oral azithromycin prophylaxis has been shown to be effective in reducing maternal and neonatal bacterial colonisation and clinical infections in The Gambia. However, it was also associated with the emergence of azithromycin resistant bacterial species especially for *S. aureus* an important pathogen responsible for both maternal and neonatal infections leading to high morbidity and mortality in The Gambia.

The aims of this PhD are to investigate the long-term impact of such intervention on *S. aureus* colonisation and antimicrobial resistance, molecular epidemiology of azithromycin resistant *S. aureus* as well as the prevalence of staphylococcal nasopharyngeal macrolide resistant genes among mother and babies following the intervention.

2.2 Objectives

The specific objectives are as follows:

1) To determine a point prevalence of nasopharyngeal carriage and azithromycin resistance for *S. aureus* at the age of 12 months among children whose mothers participated in the PregnAnZI 1 trial.

2) To characterise the population of azithromycin resistant *S. aureus* recovered from mothers and babies using genomics to identify the genetic markers of azithromycin resistance; and also explore the potential roles of clonal replacement and transmission of azithromycin resistant *S. aureus* at an individual subject level.

3) To evaluate the prevalence of nasopharyngeal macrolide resistant genes during infancy induced by intrapartum azithromycin among infants in the azithromycin arm compared to those in the placebo arm.

CHAPTER 3

3.0 METHODS

3.1 Media preparation

3.1.1 Mannitol Salt agar

Mannitol salt agar (MSA) is both a selective and differential medium used in the isolation of *Staphylococc*i. It contains 7.5% sodium chloride and thus selects for those bacteria which can tolerate high salt concentrations. MSA also distinguishes bacteria based on the ability to ferment the sugar mannitol, the only carbohydrate present in the medium.

In preparation, 111g was dissolved in 1litre of distilled water and mixed until a homogenous mixture was obtained. The mixture was then autoclaved at 121°C for 15 min in order to sterilise the medium and then allowed to cool to a temperature of about 45°C. A 20-25ml of the ready media was then poured into Petri dish plates in an air flow safety cabinet and allowed to set. The culture media plates were stored upside down at 2-8°C to reduce chances of contamination. Prior to using the plates, they were dried in the oven at 50°C for 10min to prevent bacterial swarming on the plates. Each batch of plates undergoes quality control by testing for both sterility and growth performance using American type culture controls (ATCC) bacterial strains.

3.1.2 Blood agar (BA)/Gentamicin Blood agar (GBA)

Blood agar (BA) consists of an enriched agar base to which blood cells are added. Besides providing enrichments for the growth of fastidious pathogens, Blood agar can be used to detect haemolytic properties. Gentamicin Blood Agar (GBA) is a Columbia based blood agar supplemented with gentamicin antibiotic at a concentration of 10µg/ml. It is a selective

media for isolating *S. pneumoniae.* Gentamicin antibiotic suppresses the growth of staphylococci, group A streptococci, and Gram-negative bacilli for example *E. coli*.

In preparation, 39g of Columbia agar was added to 1 litre of distilled water, mixed thoroughly to dissolve the medium completely and then autoclaved at 121°C for 15min. The media was allowed to cool to a temperature of around 45°C. 50 ml of defibrinated sheep blood (5%) was then added to the media and mixed gently to avoid the formation of bubbles. 20-25ml of the mixture poured into petri dish in a laminar flow safety cabinet to avoid contamination. In the case of GBA, 0.5ml of gentamicin (10mg/ml) was added to media following the addition of defibrinated sheep blood prior to pouring into petri dish.

3.1.3 Mueller Hinton agar (MHA)/ Mueller Hinton agar with 5% sheep Blood

MHA is the medium of choice for antibiotics sensitivity testing for rapidly growing aerobic or facultative anaerobic bacterial pathogens such as streptococci, staphylococci, members of the enterobacteriaceae, and aerobic Gram-negative rods: e.g. *Pseudomonas spp*. MHA with 5% sheep blood is recommended for susceptibility testing of *Streptococcus pneumoniae*. The agar is non-selective and differential. It contains starch known to absorb toxins released from bacteria, so that they do not interfere with the antibiotic activity. The agar is not compacted together which allows for better diffusion of the antibiotic producing a more accurate zone of inhibition.

In the preparation, 38g of MHA powder (Oxoid, UK) was added to 1 litre of distilled water and mixed thoroughly to dissolve the medium completely without the formation of bubbles. The mixture was then autoclaved at 121°C for 15 minutes before allowing to cool to 45°C. In the case of MHA with sheep blood, 50ml of defibrinated sheep blood was added to the medium

and mixed thoroughly but gently to avoid bubbles. A volume of 20-25ml of the ready media then poured into Petri dish in laminar flow safety cabinet. The medium was then allowed to set for at least 30 minutes at room temperature before storing at 2-8^{o}C.

3.1.4 Normal saline (0.85%)

Normal Saline is a useful diluent used in laboratories to maintain cell integrity and viability. It lacks the properties that may interfere with biochemical reactions and/or antibiotic susceptibility tests when used to suspend and wash bacterial cells in the lab.

In preparation, 4.25g of salt (1 tablet Oxoid code BR053G) was dissolved in 500ml of distilled water and sterilised by autoclaving at 121°C for 15minutes.

3.2 Isolation and identification of *S. aureus* and *S. pneumoniae*

Isolation and identification of both *S. aureus* and *S. pneumoniae* were performed following standard microbiology techniques. In brief, 50 µl of thawed skim milk, tryptone, glucose, and glycerol (STGG) containing nasopharyngeal swab was dispensed onto gentamicin blood agar (GBA) (CM0331 Oxoid, UK + 5% sheep blood) and mannitol salt agar (MSA) (CM0085 Oxoid, UK) for selective isolation of *S. pneumoniae* and *S. aureus* respectively. The inoculum was streaked in four quadrants (**Figure 12**) using a sterile loop to obtain discrete colonies and then incubated.

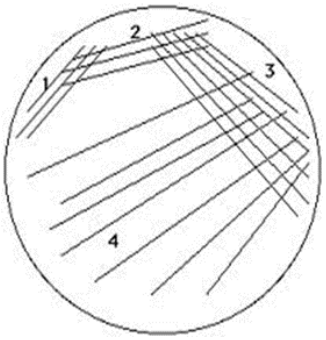


Figure 12 Streaking of the primary inoculum on to an agar plate to obtain discrete colonies

3.2.1 *Streptococcus pneumoniae*

After 20–24 hours incubation at 37 °C with 5% CO_2, the GBA plates were examined for growth and haemolysis. Morphologically distinct alpha haemolytic colonies were selected and sub-cultured on to another blood agar (BA) to obtain pure growth and screened for optochin susceptibility by measuring the zone of inhibition around the optochin disc [139]. Suspected *S. pneumoniae* isolates with optochin zones of inhibition ≥ 14 mm were confirmed to be pneumococci (**Figure 13**), those 7-13 mm were test further for bile solubility and isolates less than 7 mm were considered to be species other than pneumococci.

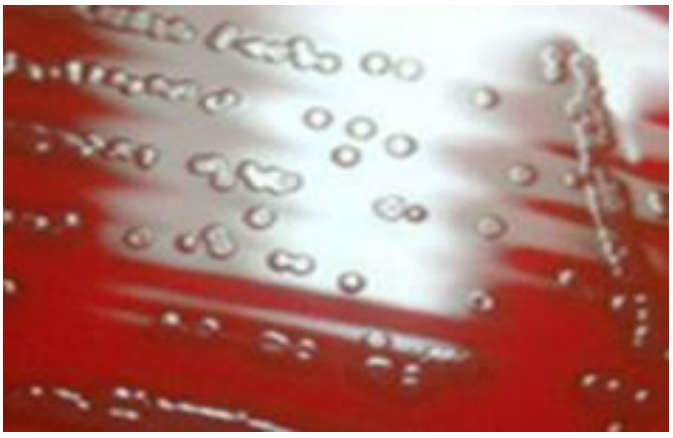


Figure 13. Confirmed *S. pneumoniae* isolate showing alpha haemolysis on a blood agar plate.

3.2.2 Bile solubility test

Bile solubility test was performed on all suspected *S. pneumoniae* isolates with optochin zone diameters measuring 7-13 mm. In the process, several colonies of the suspected *S. pneumoniae* were emulsified in a sterile bijoux bottle containing 4ml of physiological saline to obtain a turbidity equivalent to 5 McFarland standards. 2ml of the suspension was then aliquoted into a new sterile bijoux bottle. The same procedure was repeated for the *S. pneumoniae* ATCC 49619 strain for use as a positive control for the experiment. One of the bijoux bottles was marked "Test" and the other "Control". The same applies to the bijoux bottle containing the ATCC strain. Two drops of the bile reagent (sodium deoxycholate) were added to the tubes marked "Test" and two drops of distilled water were also added in the tubes marked "Control" mixed and incubated at 37°C for 2 hours. After the incubation period, the tubes were checked for clearing of turbidity. A positive bile solubility test results to clearing of the turbidity whilst the tubes containing distilled water will remain turbid after 2 hours incubation. It should be noted that the test should not be performed on old cultures, as the active enzyme called autolysin may be lost.

3.3 *Staphylococcus aureus*

Following 48 hours of incubation at 37°C, MSA plates were examined for typical *Staphylococci* colonies. Pale to golden yellow domed shaped colonies 1–2 mm in diameter (**Figure 14**) were cultured onto blood agar to obtain pure growth. A rapid latex agglutination test for the identification of *S. aureus* was performed using the Staphaurex® plus kit (Oxoid UK Cat no. OXR30950201). The test detects clumping factor, protein A and/or surface antigens characteristic of *S. aureus*. In the process, 2-3 colonies of pure suspected *S. aureus* were

collected with the aid of an applicator stick and mixed with a drop of the positive reagent and a negative reagent separately. Both mixtures were rugged for about 2 minutes. Agglutination with the positive reagent and none with negative indicates a positive test. Positive isolates were confirmed to be *S. aureus* (**Figure 14**).

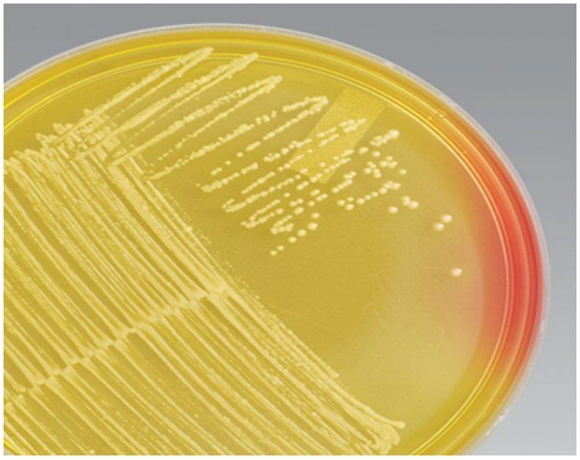


Figure 14 Confirmed *S. aureus* isolate on mannitol salt agar plate showing fermentation of mannitol resulting in the medium changing from pink to yellow colour

3.4 Antibiotic susceptibility testing

3.4.1 Disc diffusion and Epsilometry test (Etest)

Antibiotic susceptibility was performed for both *S. aureus* and *S. pneumoniae*. In brief, following the isolation and confirmation of the bacteria, a sterile loop was used to touch 3-5 well-isolated colonies of similar morphological appearance and transferred into 2.5ml normal saline until visible turbidity equal to 0.5 McFarland's Standard was obtained. A sterile swab was then immersed into the suspension and gently press on the sides of the bijou bottle to remove excess fluid. The swab then evenly streaked over the surface of the medium in three directions, rotating the plate 90 °C to ensure even distribution. Then the plate was incubated

at room temperature for 15min for the moisture to be absorbed by the medium. A disc dispenser or sterile forceps was then used to apply the antibiotic discs. The discs were placed about 15mm from the edge of the plate and less than 25mm between discs. Plates were incubated within 15-30 minutes of discs application. Resistance was initially screened using the following antibiotic discs: azithromycin (15 µg), chloramphenicol (30µg), clindamycin (2µg) and erythromycin (15µg). In addition, only *S. pneumoniae* isolates were screened for trimethoprim-sulfamethoxazole (1.25/23.75µg) and oxacillin (1µg) susceptibility and *S. aureus* for cefoxitin (30µg) susceptibility. **Table** 3 shows the cut off values used in the classification of *S. pneumoniae* and *S. aureus* as sensitive, intermediate or resistant.

Table 3 showing the Disc diffusion cut offs for the different antibiotics (CLSI 2017)

Bacteria	Antibiotic	Disc diffusion cut off(zone of growth inhibition in mm)		
S. aureus		Sensitive	Intermediate	Resistant
	Azithromycin 15 µg	≥18	14-17	≤13
	Chloramphenicol 30 µg	≥18	13-17	≤12
	Clindamycin 2 µg	≥21	15-20	≤14
	Erythromycin 15 µg	≥23	14-22	≤13
	Cefoxitin 30 µg	≥25	-	≤24
S. pneumoniae				
	Azithromycin 15 µg	≥18	14-17	≤13
	Chloramphenicol 30 µg	≥21	-	≤20
	Clindamycin 2 µg	≥19	16-18	≤15
	Erythromycin 15 µg	≥21	16-20	≤15
	Trimethoprim-sulfamethoxazole 1.25/23.75µg	≥19	16-18	≤15
	Oxacillin 1µg	≥20	-	-

All isolates that were assessed as of intermediate or resistant by the disc diffusion method were tested further using the E-test for confirmation except those isolates resistant to cotrimoxazole. For these isolates, only a random selection of 90 were confirmed by the E-test due to lack of funds. Sample preparation for E-test was performed exactly as described above for the disc diffusion method. However, only a single E-test strip was added per plate prior to overnight incubation. After the incubation period a symmetrical inhibition ellipse was

produced by the strip on the plate. The MIC value is read from the scale in terms of µg/mL where the ellipse edge intersects the strip. Susceptibility to the different antibiotics was determined following the clinical and laboratory standards institutes guidelines (CLSI) **Table 4** [141].

Table 4 Showing the E-test cut offs for the different antibiotics (CLSI 2017)

Bacteria	**Antibiotic**	**E-test cut-off**		
S. aureus		Sensitive	Intermediate	Resistant
	Azithromycin 15 µg	≤2	4	≥8
	Chloramphenicol 30 µg	≤8	-	≥16
	Clindamycin 2 µg	≤0.5	1-2	≥4
	Erythromycin 15 µg	≤0.5	1-4	≥8
	Cefoxitin 30 µg	≤4	-	≥8
S. pneumoniae				
	Azithromycin 15 µg	≤0.5	1	≥2
	Chloramphenicol 30 µg	≤4	-	≥8
	Clindamycin 2 µg	≤0.25	0.5	≥1
	Erythromycin 15 µg	≤0.25	0.5	≥1
	Trimethoprim-sulfamethoxazole (1.25/23.75µg)	≤0.5/9.5	1/19-2/38	≥4/76
	Oxacillin (1µg)	≤2	4	≥8

3.4.2 Vitek 2

The VITEK 2 is an automated microbiology system that utilizes a growth-based technology. The system accommodates colorimetric reagent cards that are incubated and interpreted automatically. The card has 64 wells that is barcoded with information on card type, expiration date, lot number and unique card identification number. Different test kits are available including ID-GN (Gram-negative bacillus identification), ID-GP (Gram-positive cocci identification), AST-GN (Gram-negative susceptibility) and AST-GP (Gram-positive susceptibility). The Vitek 2 ID-GP card identifies 124 species of staphylococci, streptococci, enterococci and a select group of Gram-positive organisms within 8 hours or less.

All *S. aureus* and *S. pneumoniae* isolates found to be resistant by E-test to azithromycin, erythromycin, or clindamycin were retested using the VITEK-2 (bioMérieux, France). The VITEK-2 results were considered definitive. In the process, a new virtual cassette was selected and the barcode for a Gram-positive cassette was scanned followed by entry of sample number in the accession number column. The organism ID name was selected from the drop-down list in the organism field. A 0.5-0.63 McFarland suspension was prepared in 0.45% sterile saline. Following this 280µl of the suspension was aliquoted and dispensed into another tube containing 3ml of 0.45% sterile saline. The AST card was then turned around so that the filler tube was directly in contact with the suspension. The complete unit was then inserted in the Vitek 2 machine first into a chamber where the suspension was loaded into the cassette and then into another chamber were the susceptibility test was performed. The procedure was repeated for all the samples with a maximum of ten samples per cassette and the entries saved. The results of the susceptibility testing for the antibiotic were retrieved from the computer the next day **Figure 15.**

Figure 15 Vitek 2 machine with computer sourced from https://www.dksh.com/media/953/997/3da2e-vitek2-compact-biomerieux-,0.png for antibiotic susceptibility and analysis of the results

3.5 DNA extraction

3.5.1 DNA extraction from pure *S. aureus* culture

Genomic DNA was extracted using the Sigma GenElute™ Bacterial Genomics DNA Kit NA2120 1KT with some slight modifications. A single isolated *S. aureus* colony was introduced in to 10ml of brain heart infusion broth (BHI) and incubated overnight. Following incubation in BHI broth, 0.5ml of the cell culture was dispensed into an eppendorf tube. The tube was then centrifuged for 1min @ 13,000 x g. This was followed by washing in 1ml phosphate buffered saline PBS and centrifuged for 2min @ 5,000 g. The cells were then suspended in 200µl of

lysis buffer comprising 200µl of enzymatic lysis buffer (20mM Tris-HCl pH 8.0, 2mM sodium EDTA and 1.2% Triton x-100), 9mg of lysozyme and 5µl of lysostaphin 2mg/ml. The mixture was incubated in a 37°C water bath for 20min. 20µl of RNAse A was added and incubated for further 10minutes. 20 µl of the Proteinase K solution (20mg/ml) was added to the sample, followed by 200 µL of Lysis solution C (B8803). The mixture was vortexed thoroughly (about 15 seconds) and incubated at 55 °C for 10 minutes. 200 µl of ethanol (95–100%) was then added to the lysate and mixed thoroughly by vortexing for 5–10 seconds to obtain a homogeneous mixture. The entire contents of the tube were then transferred into the binding column and centrifuged at ≥ 6500 × g for 1 minute. The collection tube containing the eluate was discarded and the column placed in a new 2 ml collection tube. The columns were washed twice using wash solution 1 and 2 respectively. 200 µl of the Elution Solution (B6803) was directly added onto the centre of the column and incubated at room temperature for 5 min before centrifuging for 1 minute at ≥ 6500 × g to elute the pure DNA.

3.5.2 Extraction of total nucleic acid from NPS

Total nucleic acid was extracted directly from NPS using QIAamp DNA Mini Kit (QIAGEN, United Kingdom) protocol with some modifications. Frozen nasopharyngeal swabs in STGG medium were initially thawed at room temperature, vortexed briefly and 200µl of the sample added to 200µl of lysis buffer (TE buffer, lysozyme 30mg/ml, mutanolysin 50U/ml, lysostaphin 200µg/ml) before being incubated at 37°C for 1 hour. AL buffer and proteinase K were then added and incubated further at 56°C for 1 hour. After the second incubation, 250µl of ethanol was added and the whole suspension transferred into a spin column and centrifuged at 8000

rpm for 1 minute. The columns were washed twice before eluting pure total nucleic acid in 100µl of the elution buffer and stored at -20°C until use.

3.6 Nano Drop

Following extraction of DNA or total nucleic acid, Nanodrop was performed on all of the samples in order to obtain the nucleic acid yield but also the purity of the DNA extract. In the process, the nanodrop software was opened on the computer by clicking the icon ND100 and nucleic acid was selected. The sensor pedestal cleaned and 1ul of RNAse free water added without generating bubbles to initialise the equipment. This was followed by blanking the equipment by adding 1ul of the elution buffer to the sensor pedestal. Then, the number of each sample was entered ensuring DNA was selected as sample type. The ratios 260/280 (1.8-2.1) and 260/230 (1.8-2.0) were used to determine protein and organic contamination of sample respectively.

3.7 Qubit assay

The Qubit assays use target-selective dyes that emit fluorescence when bound to DNA, RNA or protein. It is less prone to over estimation of sample concentration due to contaminants such as salts, solvents, detergents, proteins, free nucleotides. The Qubit was used to more accurately determine concentration of DNA extracted from pure *S. aureus* cultures during the library prep stage of the whole genome sequencing. In the process, 1µl of Qubit reagent was added to 199µl of Qubit buffer to obtain the working solution. Following the preparation of the working solution for the total number of samples and the two standards, 10µl of standard 1 then standard 2 were each added to 190µl of the Qubit working solution, vortexed briefly

and incubated at room temperature for 2min before being read. Following calibration, 198µl of Qubit working solution and 2µl each sample were added, incubated for 2min and concentration measured in ng/µl **Figure 16.**

Figure 16 The Qubit assay and the reagents(buffers and standards) sourced from https://www.thermofisher.com/content/dam/LifeTech/global/life-sciences/images/qubit-assays.png for DNA quantification

3.8 Cloning and transforming *ermC* and *msr(A)* genes

Vector pRAB11 was initially amplified with the following primers to create a backbone for SLiCE (seamless ligation cloning extract) cloning using the following primer pairs: pRAB11_F and pRAB11_R. The *ermC*_2/13 and *msr*(*A*) genes were also amplified using ermC_pRAB11_F *ermC*_pRAB11_R and *msr(A)*_Saur_pRAB_F msr(A)_Saur_pRAB_R primers respectively. The

primer sequences are shown in **Table 5**. The PCR fragments were ligated using SLiCE and transformed into IM08 competent *E. coli* cells. The transformed cells were plated on Luria broth (LB) agar with 100µg/ml ampicillin and incubated overnight at 37°C. Successful transformation was confirmed by colony PCR from colonies growing on the LB agar. The confirmed colonies were cultured overnight, and plasmids extracted using QIAGEN mini prep kit. Plasmids carrying *ermC* or *msr*(*A*) gene were electroporated into an azithromycin sensitive *S. aureus* strain (S70065MN00) and plated on LB agar with 10µg/ml chloramphenicol before incubating overnight. Colonies were screened to confirm the presence of either *ermC* or *msr*(*A*) genes using the above primers.

Table 5 Showing the primer sequences for the vectors and macrolide resistant genes

Primer name	**Primer sequence**
pRAB11_F	**GGTACCCAGCTTTTGTTCCCTTTAGTGAGGGAATTCACTGGCCGTCGTTTTACA**
pRAB11_R	**GAGCTCCAATTCGCCCTATAGTGAGTCGAGATCTGTTAACGGTACCATCA.**
ermC_pRAB11_F	**CGACTCACTATAGGGCGAATTGGAGCTCTATAACATAAAATCGTCTACAAATAG**
ermC_pRAB11_R	**CCTCACTAAAGGGAACAAAAGCTGGGTACCATGCAGTTTATGCATCCCTTAAC**
msr(A)_Saur_pRAB_F	**CGACTCACTATAGGGCGAATTGGAGCTCACAATTATCTCCTTTTTAATTATTTAGA**
msr(A)_Saur_pRAB_R	**CCTCACTAAAGGGAACAAAAGCTGGGTACCGAAATATTCAATATAAATTAGTGTATAGTTT**

3.9 Bacterial conjugation

The donor (S80062MN28; azithromycin MIC 192 µg/ml, rifampicin MIC <1 µg/ml; fusidic acid MIC <1 µg/ml) and recipient strain that was induced to high level rifampicin and fusidic acid resistance through serial passage (S70065MN00, azithromycin MIC 1 µg/ml, rifampicin MIC >32 µg/ml; fusidic acid MIC >32 µg/ml) were mixed on a membrane filter (Millipore) with a donor-to-recipient ratio of 1:1. Plates were incubated overnight at 37°C. The bacterial cells were resuspended in PBS and spread on BHI agar plates containing the appropriate antibiotics. To quantify conjugative transfer efficiencies, dilutions were plated on BHI media containing antibiotics for the selection of; donor cells, recipient cells and plasmid containing recipient cells. Trans-conjugants were selected on plates containing azithromycin, rifampicin and fusidic acid all at 32 µg/ml. All conjugation assays were performed in duplicates. Control conjugation experiments were performed with the donor strain only, and no spontaneous resistant colonies were observed. The resistance profile of donor, recipient and transconjugant are summarized in **Table S1**.

3.10 PCR

The primers for the amplification of the macrolide resistant genes [*msr(A)_F ATCCAATCATTGCACAAAATCTAACATT, msrA_R TAAATAGCTTCAAGTAAAGTTGTCTTACC* and *erm*C_F CTTGTTGATCACGATAATTTCCAAG, *ermC*_R TTGTATTCTTTGTTAACCCATTTCATAAC] and a *S. aureus* thermostable nuclease *nuc* gene [*nuc*_F GCGATTGATGGTGATACGGTT nuc_R AGCCAAGCCTTGACGAACTAAAGC] were designed using an online primer design tool (Primer3plus http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and synthesised by Metabion, Germany. Primers sets were diluted to a final concentration of

10µM and assayed in 20µl PCR reaction volume containing 1µl of each primer, 10µl 2x Phire Green Hot Start II PCR (Thermo Scientific, United Kingdom) reaction buffer, 7µl nuclease water and 1µl of the sample. The cycling conditions included an initial denaturation at 98°C for 30 seconds, 35 cycles of denaturation at 98°C for 5 seconds, annealing at 52°C for 5 seconds and extension at 72°C for 10 seconds with a final extension at 72°C for 1 minute. Fully sequenced *S. aureus* carrying either the *erm*C or *msr(A)* genes were used as positive control.

3.11 Electrophoresis

The PCR products were analysed using QIAxcel advanced Screen gel 1.5.0 (QIAGEN, UK) **Figure 17**. The QIAxcel enables separation and analysis of a variety of nucleic acids including single or multiple PCR fragments, DNA digested with restriction endonucleases, synthesized oligonucleotides, total RNA and cRNA. The setup allows the user to define run parameters and preselect DNA size markers.

For the analysis of amplified PCR products of the gene targets, a tolerance rate of ±15% of the expected band sizes [(*msr(A)* 145bp, *erm*C 398bp and *nuc* 279bp)] and DNA size markers (1000bp and 15bp) were used. The relative fluorescence unit (RFU) was determined to be low (≤1.00 RFU) or high (>1.00 RFU) for all samples positive for the gene target.

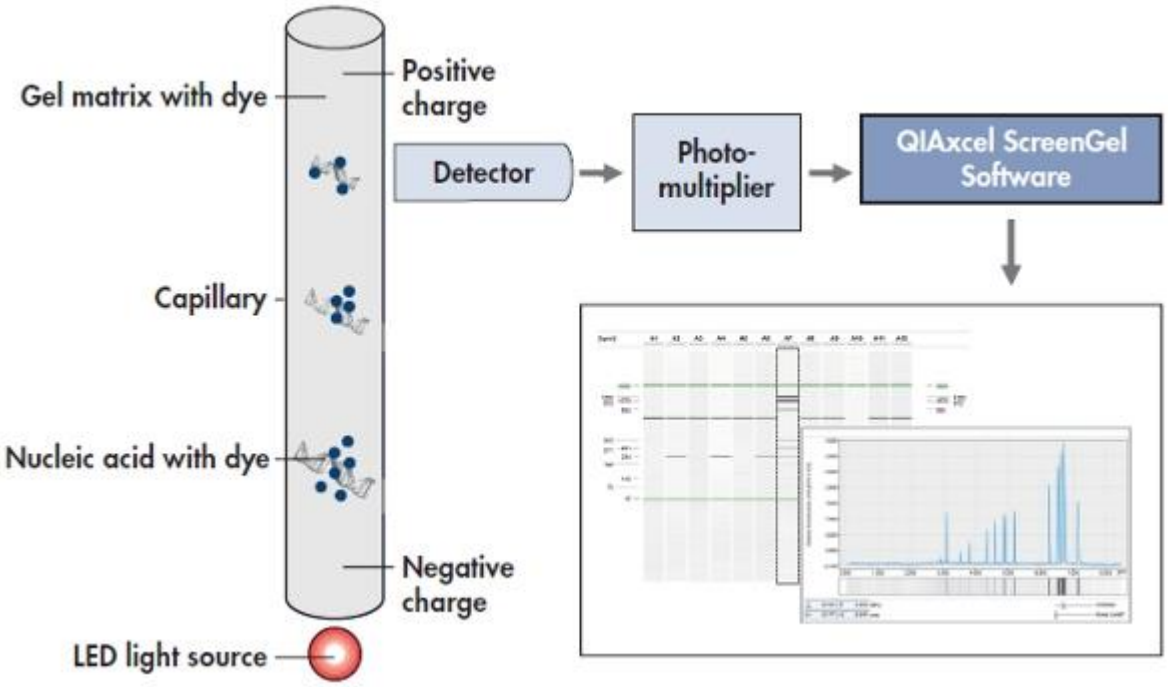


Figure 17 The QIAxcel procedure for the separation of nucleic acid molecules, detection, photomultiplication before conversion of the data to an electropherogram and gel image by QIAxcel ScreenGel software reproduced from https://media.americanlaboratory.com/m/20/article/142596-fig1.jpg

3.12 Whole genome sequencing and bioinformatic analysis

Staphylococcus aureus genomic DNA was extracted using the Sigma GenElute™ Bacterial Genomics DNA Kit NA2120 1KT. DNA libraries were prepared using the Nextera XT kit (Illumina, San Diego, CA, United States), and sequenced on the Illumina Nexseq platform using 2 × 150-bp chemistry. The raw reads were initially quality controlled to obtain information such as minimum and maximum read lengths, GC content, average quality and depth of coverage. Short-read sequence data was assembled using SPAdes v3.11.1 [142], excluding the pre-assembly error correction, including a post-assembly error correction and a minimum coverage threshold of "5" to remove contaminants. A hybrid assembly approach using both long- and short-read sequence data was utilised to assemble isolate S80062MN28, performed using unicycler v0.4.6 with the assembly mode set as "bold"[143]. Annotation of plasmid pS80062MN28 was performed using prokka v1.13.3[144], preferentially annotating from plasmid pJSA01 (accession AP014922.1)[145]. Comparative alignment of plasmid sequences was performed using the Artemis Comparison Tool v17.0.1, excluding matches ≤ 100 bp in length[146]. *In silico* MLST and antibiotic resistance gene detection was performed on draft assemblies and complete genomes using mlst v2.10 (https://github.com/tseemann/mlst) and abricate v0.8.10 (https://github.com/tseemann/abricate), using the NCBI antimicrobial resistance database (ncbi: updated 20-Sept-2018) and the virulence factor database (vfdb: updated 14-Aug-2018).

For the phylogenetic analysis, short-read sequence data was mapped to the complete genomes of six *S. aureus* reference genomes (genome accessions: CP026964.1 [ST1]; CP026968.1 [ST5]; CP012970.1 [ST8]; LS483319.1 [ST15]; CP024998.1 [ST152]; LS483314.1 [ST669]), each selected for being the most closely related complete genome for each ST

identified in the study population. Read mapping and calling of single nucleotide polymorphisms (SNPs) was completed using snippy v4.3.5 (https://github.com/tseemann/snippy). Whole genome alignments were used to construct maximum likelihood (ML) phylogenetic trees with iqtree v1.6.5 [147]. Model selection was used to find the best nucleotide substitution model for each alignment (chosen according to the Bayesian Information Criterion): this was either the HKY+F for ST5 and ST15 [148], or the F81+F for ST1, ST8, ST152 and ST669 [149]. Alignments composed of greater than 5 isolates (ST5 and ST15) were subjected to 1000 ultra-fast bootstrap replicates [150]. ML trees were visualized using FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). Core pairwise SNP distances were calculated using an in-house pearl script.

The global *S. aureus* phylogeny was constructed using publicly available genomes and metadata as described in Guerillot *et al.* [151]. The 66 isolates sequenced as part of this study were added, together with 28 newly sequenced *S. aureus* genomes from Africa (downloaded from the Staphopia database [152]. The 7191 genomes were mapped to the fully assembled reference genome *S. aureus* NRS384 and core genome alignment of the global collection (n=7192), all CC5 isolates (n=2735) or all CC15 isolates (n=179) were generated with snippy v4.3.6. Maximum likelihood phylogenetic trees were inferred with Fast Tree v2.1.8 using the generalized time reversible (GTR) model. Trees were plotted and annotated using the R library *ggtree* [153]. Sub-trees of CC5 and CC15 were generated from the full CC5 or CC15 phylogenetic trees by extracting only isolates with a tip distance of <23 nodes from the isolates sequenced in this study.

3.13 Phenotypic testing of transformed *S. aureus*

A 0.5M suspension was prepared for each of the wild type (S70065MN00), wild type + empty plasmid and wild type + mutants in normal saline. The suspension was streaked on to Mueller Hinton agar using a sterile swab before placing an azithromycin E-test strip and incubating for 20 hours at 37°C.

3.14 Data management and statistical analysis

Laboratory results and other personal data including randomization number, date samples were collected, lab staff identification were transcribed onto bar-coded forms and submitted to data management for entry. The data were entered independently by two data entry clerks in *OpenClinica* (www.openclinica.com) and antibiotic susceptibility data for *S. pneumoniae* were stored in a REDcap™ database. The data base was then cleaned and verified to ensure data quality.

In the analysis, each of the following antibiotics: azithromycin, chloramphenicol, clindamycin, erythromycin, trimethoprim-sulfamethoxazole, oxacillin and cefoxitin the resistant bacterial carriage prevalence was compared between trial arms using the chi-square test. This was done for both *S. pneumoniae* and *S. aureus*. In addition, I assessed if azithromycin resistance up to day 28 post-treatment was associated with resistance 12 months later. For this analysis, only children who were sampled during all five time points in the trial (day 0, 3, 6, 14, 28) and were also sampled in the 12-month CSS were included.

Fisher exact test was used to compare the prevalence of sequence types or macrolide resistance genes between the azithromycin and placebo group. A P value of 0.05 was used as the cutoff for statistical significance.

Pearson's χ2 test was used to compare the prevalence and relative fluorescence of macrolide resistant genes between arms at birth, at day 28 and at 12 months. The χ2 test was also used to test for an association between the resistance genes at day 28. This analysis was stratified by trial arm, and a Mantel-Haenszel test was used to test for interaction. P-value ≤ 0.05 was used as the cut off for statistical significance. All analyses were done using STATA/SE v12.1 (https://www.stata.com/).

CHAPTER 4

4.1 PhD Manuscript #1

4.2 LONG-TERM IMPACT OF ORAL AZITHROMYCIN TAKEN BY GAMBIAN WOMEN DURING LABOR ON PREVALENCE AND ANTIBIOTIC SUSCEPTIBILITY OF STREPTOCOCCUS PNEUMONIAE AND STAPHYLOCOCCUS AUREUS IN THEIR INFANTS: FOLLOW-UP OF A RANDOMIZED CLINICAL TRIAL

Published 28th March 2018 in the ***Clinical Infectious Disease Journal***

Clinical Infectious Diseases

MAJOR ARTICLE

Long-term Impact of Oral Azithromycin Taken by Gambian Women During Labor on Prevalence and Antibiotic Susceptibility of *Streptococcus pneumoniae* and *Staphylococcus aureus* in Their Infants: Follow-up of a Randomized Clinical Trial

Abdoulie Bojang,[1] Bully Camara,[1] Isatou Jagne Cox,[1] Claire Oluwalana,[1] Kodou Lette,[1] Effua Usuf,[1,2] Christian Bottomley,[3] Benjamin P. Howden,[4] Umberto D'Alessandro,[1,2,5] and Anna Roca[1,6]

[1]Medical Research Council Unit The Gambia at London School of Hygiene and Tropical Medicine, Fajara; [2]Faculty of Infectious and Tropical Diseases and [3]Medical Research Council Tropical Epidemiology Group, London School of Hygiene and Tropical Medicine, United Kingdom; [4]Microbiological Diagnostic Unit Public Health Laboratory, Department of Microbiology and Immunology, University of Melbourne, Doherty Institute for Infection and Immunity, Victoria, Australia; [5]Institute of Tropical Medicine, Antwerp, Belgium; and [6]Faculty of Epidemiology and Population Health, London School of Hygiene and Tropical Medicine, United Kingdom

Background. Oral azithromycin given to women in labor decreases maternal and neonatal bacterial carriage but increases azithromycin-resistant bacteria during at least 4 weeks following the intervention. We assessed the prevalence of bacterial carriage and azithromycin resistance 12 months after treatment among study infants.

Methods. Nasopharyngeal swabs (NPSs) were collected between November 2014 and May 2015 from children aged 11–13 months whose mothers had received azithromycin or placebo during labor. *Streptococcus pneumoniae* and *Staphylococcus aureus* were iso-lated using conventional microbiological methods. Antibiotic susceptibility was determined by disk diffusion and confirmed by Etest or VITEK-2.

Results. NPSs were collected from 461 children. The prevalence of *S. pneumoniae* and *S. aureus* was similar between chil-dren from the azithromycin and placebo arms (85.0% vs 82.1%; odds ratio [OR], 1.23 [95% confidence interval {CI}, .73–2.08] for *S. pneumoniae* and 21.7% vs 21.3%; OR, 1.02 [95% CI, .64–1.64] for *S. aureus*). Prevalence of azithromycin-resistant *S. pneumo-niae* was similar in both arms (1.8% vs 0.9% in children from the azithromycin and placebo arms, respectively; OR, 2.10 [95% CI, .30–23.38]); resistance to other antibiotics was also similar between arms. For *S. aureus*, there was no difference in azithromycin resistance between children in the azithromycin (3.1%) and placebo (2.6%) arms (OR, 1.22 [95% CI, .35–4.47]) or resistance to any other antibiotics.

Conclusions. The higher prevalence of *S. aureus* azithromycin resistance observed among women treated during labor and their babies 4 weeks after treatment had waned 12 months after delivery. Azithromycin intervention did not induce other antibiotic resistance to *S. pneumoniae* or *S. aureus*.

Clinical Trials Registration. NCT01800942.

Keywords. azithromycin; *S. aureus*; *S. pneumoniae*; resistance; West Africa.

Azithromycin is a second-generation broad-spectrum macrolide used to treat infections such as pneumonia, middle ear infections, and sexually transmitted infections [1, 2]. Azithromycin has also been used in mass drug administra-tion (MDA) campaigns to control trachoma in several endemic countries in Africa [3–6]. The impact of these MDA campaigns has varied from one country to another [6–8], but when the baseline prevalence of trachoma is low, 1 round of MDA with azithromycin (MDA-Z) is sufficient to eliminate trachoma [3]. Furthermore, MDA-Z may have beneficial effects beyond tra-choma control. In rural Gambia, 3 annual rounds of MDA-Z reduced by 24.2% asymptomatic pneumococcal carriage for at least 1 month [9] and, in Ethiopia, it reduced by 49% all-cause mortality in children aged 1–9 years [10].

Azithromycin has also been given in combination with sulfadoxine-pyrimethamine (SP) during pregnancy with the aim of reducing the prevalence of low birth weight. In Malawi and Papua New Guinea, babies born to mothers who received azith-romycin and SP had a reduced risk of low birth weight [11, 12].

Received 17 November 2017; editorial decision 18 March 2018; accepted 27 March 2018; published online March 28, 2018.

Correspondence: A. Roca, Medical Research Council Unit The Gambia, London School of Hygiene and Tropical Medicine, PO Box 273, Fajara, The Gambia (aroca@mrc.gm).

Clinical Infectious Diseases® 2018;67(8):1191–7

 DOI: 10.1093/cid/ciy254

MDA-Z may increase the prevalence of macrolide resistance, even after the administration of a single dose. In The Gambia, MDA-Z resulted in a short-term increase in azithromycin-re-sistant *Streptococcus pneumoniae* [9], while in Tanzania a single dose of MDA-Z increased prevalence of resistance for >6 months [13]. The prevalence was also high in Ethiopia among children after receipt of several annual doses [14] and declined after azithromycin was discontinued [15].

For *Staphylococcus aureus*, another common bacterial pathogen, there are increasing data on the effect of MDA-Z on resistance. In rural Gambia [3], 3 annual rounds of MDA-Z were associated with a long-term (30 months after the first round MDA-Z) increase in both the prevalence of azithromycin-resistant and inducible macrolide lincosamides and streptogramin B (MLS_B)–resistant *S. aureus* [16]. In Papua New Guinea, the proportion of azithromycin-resistant *S. aureus* was 5 times higher among pregnant women treated with azithromycin than in those in the control group [17].

In a recent trial in The Gambia, administering 2 g of oral azithromycin during labor reduced significantly maternal and neonatal nasopharyngeal carriage of *S. pneumoniae*, *S. aureus*, and group B *Streptococcus* [6], as well as maternal and neonatal infections [18, 19]. Four weeks after birth, children in the intervention arm had similar prevalence of nasopharyngeal carriage of *S. pneumoniae* azithromycin resistance compared to those in the control arm (2.1% vs 2.2%; $P = 1.000$), but higher prevalence of *S. aureus* azithromycin resistance (4.5% vs 16.7%; $P < .001$) [20]. To determine the persistence of azithromycin resistance among study children, we collected nasopharyngeal swabs (NPSs) at their first birthday.

MATERIALS AND METHODS

Study Site and Population

Trial participants were recruited from the Bundung Maternal and Child Health Hospital, formerly called Jammeh Foundation for Peace, a government-run health center located in western Gambia that manages on average 4500 deliveries per year [21]. The population covers the main ethnic groups in The Gambia with a high illiteracy rate. The climate of the area is typical of the sub-Sahel region.

Main Trial

Details of the study protocol have been described elsewhere [21]. In brief, this was a phase 3, double-blind, placebo-controlled trial where 829 pregnant women attending the labor ward in the study health facility were randomized to receive a single oral dose of 2 g of either oral azithromycin or placebo (ratio 1:1). The trial started in April 2013 and lasted 14 months (12-month recruitment period and 2 additional months of follow-up). Study participants (women and their newborns) were monitored for 8 weeks after the intervention. Neonatal NPSs were collected during the first 4 weeks of the follow-up as part of the trial. Prevalence of nasopharyngeal carriage of *S. pneumoniae* was significantly lower during the entire neo-natal period among neonates exposed to azithromycin (at day 28: 37.2% vs 24.8%; prevalence ratio [PR], 0.67 [95% confi-dence interval {CI}, .53–.83]; $P < .001$). Prevalence of carriage of *S. pneumoniae*–resistant strains was low throughout and sim-ilar between arms (at day 28: 2.1% vs 2.2%; PR, 1.04 [95% CI, .40–2.75]; $P = 1.000$). For *S. aureus*, prevalence of nasopharyngeal carriage peaked at day 6 and by day 28 was still significantly lower in the azithromycin arm (35.3% vs 25.6%; PR, 0.73 [95% CI, .58–.91]; $P = .005$), whereas prevalence of azithromycin-re-sistant *S. aureus* carriage peaked at day 28 (4.5% vs 16.7% in the azithromycin and placebo arms; PR, 3.68 [95% CI, 2.19–2.75]; $P < .001$) [20].

Cross-sectional Survey

Between November 2014 and May 2015, children aged 12 months (range, 11–13 months) whose mother had partic-ipated in the main trial were visited at home and had an NPS collected. Consent was obtained by a trained nurse blinded to the treatment allocation.

Ethical Approval

Both the main trial and the cross-sectional survey (CSS) were approved by the Joint Medical Research Council/The Gambia Government Ethics Committee. Mothers of children included in the CSS signed an additional informed consent.

Sample Handling

The NPS was collected using calcium alginate (Expotech USA) swab as described previously [21]. In brief, each swab was immediately placed into a vial containing skim milk-tryp-tone-glucose-glycerol transport medium and then into a cold box before being transported to the laboratory within 8 hours [22]. Upon receipt, the tubes were vortexed for a minimum of 20 seconds before being stored in freezers at –70°C.

Laboratory Methods

Details of the laboratory methods were described previously [21]. In brief, 50 µL of the sample was dispensed onto gentamicin blood agar (GBA) (CM0331 Oxoid, United Kingdom, plus 5% sheep blood) and mannitol salt agar (MSA) (CM0085 Oxoid, United Kingdom) for selective isolation of *S. pneumoniae* and *S. aureus*, respectively.

Streptococcus pneumoniae

After 20–24 hours of incubation at 37°C with 5% carbon dioxide, GBA plates were examined for typical α-hemolytic colonies. Morphologically distinct α-hemolytic colonies were selected and subcultured on another blood agar to obtain pure growth and screened for optochin susceptibility [22]. *Streptococcus pneumoniae* isolates were confirmed as described previously [21].

Staphylococcus aureus

Following 48 hours of incubation at 37°C, MSA plates were exam-ined for typical staphylococci colonies. Pale to golden yellow dome-shaped colonies 1–2 mm in diameter were cultured onto blood agar to obtain pure growth. A coagulase test was performed on all suspected colonies using the Staphaurex plus kit (Oxoid, United Kingdom, catalog number OXR30950201). Isolates testing positive for coagulase were confirmed to be *S. aureus.*

Antibiotic Susceptibility Testing

Antibiotic susceptibility was performed as described previously [21]. In brief, resistance was initially screened by disk diffusion method. Both *S. pneumoniae* and *S. aureus* isolates were screened using the following antibiotic discs: azithromycin (15 µg), chloramphenicol (30 µg), clindamycin (2 µg), and erythromycin (15 µg). In addition, only *S. pneumoniae* isolates were screened for trimethoprim-sulfamethoxazole (1.25/23.75 µg) and oxa-cillin (1 µg), and *S. aureus* isolates were screened for cefoxitin (30 µg). All isolates that were of intermediate or resistant by the disk diffusion method were further tested using the Etest for confirmation except those isolates resistant to cotrimoxazole. Due to limited resources, only 90 of these isolates were ran-domly selected to be confirmed by the Etest. Susceptibility to the different antibiotics was determined following the Clinical and Laboratory Standards Institute guidelines [23]. In addition, D-test was performed on all macrolide (azithromycin and/ or erythromycin)–resistant clindamycin-sensitive isolates to assess inducible clindamycin resistance [24]. In an additional effort to confirm resistance, all *S. pneumoniae* isolates found to be resistant by Etest to azithromycin, erythromycin, or clinda-mycin were retested using the VITEK-2 (bioMérieux, France). The VITEK-2 results were considered definitive.

Table 1. Comparison of Children in the 12-Month Survey With Those Who Did Not Participate

Variable	12-Month Survey, no./No. (%)		*P* Value
	Included	Not Included	
Sex			
Female	230/465 (49.5)	175/378 (46.3)	.360
Male	235/465 (50.5)	203/378 (53.7)	
Ethnicity			
Mandinka	201/462 (43.5)	151/361 (41.8)	.367
Wollof	49/462 (10.6)	45/361 (12.5)	
Jola	80/462 (17.3)	47/361 (13.0)	
Fula	76/462 (16.5)	68/361 (18.8)	
Other	56/462 (12.1)	50/361 (13.9)	
Maternal age at delivery, y			
18–19	30/465 (6.5)	37/378 (9.8)	.109
20–29	295/465 (63.4)	244/378 (64.6)	
≥30	140/465 (30.1)	97/378 (25.7)	
Multiple pregnancies			
No	453/465 (97.4)	362/372 (95.8)	.183
Yes	12/465 (2.6)	16/378 (4.2)	

Data Management and Statistical Analysis

Laboratory data were transcribed onto bar-coded forms and submitted to data management for entry. The data were double entered in OpenClinica (www.openclinica.com), and antibiotic susceptibility data for *S. pneumoniae* were stored in a REDcap database.

For each antibiotic, the resistant bacterial carriage prevalence was compared between trial arms using the χ^2 test. This was done for both *S. pneumoniae* and *S. aureus*. In addition, we assessed if resistance up to day 28 posttreatment was associated with resistance 12 months later. For this analysis, only children who were sampled during all 5 time points in the trial (days 0, 3, 6, 14, and 28) and were also sampled in the 12-month CSS were included. All analyses were carried out using Stata version 12.0 software.

RESULTS

Study Population

Among 814 study children alive at the end of the initial trial period (8 weeks of age), 5 had died and 196 were >13 months at the time of the CSS survey. Among the remaining 613 children, 465 (76.0%) were enrolled in this study and NPSs were collected from 461 of them (99.1%). Demographic characteristics were similar between children included in the CSS and those who were not (Table 1). Baseline characteristics (sex, ethnic group, season at birth, maternal age, and age at follow-up) in the azi-thromycin (n = 226) and the placebo groups (n = 235) were similar, except for a higher number of multiple pregnancies in the placebo arm (*P* = .037; Table 2).

Prevalence of Bacterial Carriage

The prevalence of *S. pneumoniae* carriage was high and similar between arms (85.0% vs 82.1% in the azithromycin and placebo arms, respectively; odds ratio [OR], 1.23 [95% CI, .73–2.08]; Table 3). The prevalence of *S. aureus* carriage was lower but also similar between arms (21.7% vs 21.3% in the azithromycin and placebo arms respectively; OR, 1.02 [95% CI, .64–1.64]).

Prevalence of Antibiotic Resistance

Streptococcus pneumoniae

Only 6 of the 385 *S. pneumoniae* isolates were resistant to azithromycin, and prevalence was similar between study arms (1.8% vs 0.9% in the azithromycin and placebo arms, respectively; OR, 2.10 [95% CI, .30–23.38]). Prevalence of *S. pneumoniae* resistance against the other antibiotics tested was also similar between arms (Table 4). D-test was performed on 14 samples (6 azithromycin and 8 erythromycin resistant) and all were nega-tive. Resistance to cotrimoxazole based on disk diffusion was the highest among all the antibiotics tested (Table 4). Among the cotrimoxazole-resistant isolates, 30% were retested using Etest and 63.3% (57 of 90) were confirmed. All *S. pneumoniae* isolates were susceptible to at least 3 of the antibiotics tested,

Table 2. **Baseline Characteristics of Mothers and Children in the 2 Groups**

Characteristic	Azithromycin Group (n = 226)	Placebo Group (n = 235)	*P* Value
Maternal age at delivery, mean (SD)	26.5 (5.3)[a]	26.6(5.0)	.684
Mode of delivery			
Vaginal	220 (97.4)	233(99.2)	.165
Cesarean	6 (2.7)	2(0.9)	
Information at birth			
Multiple pregnancy	2 (0.9)	10(4.3)	.037
Sex[b]			
Female	117 (51.8)	104(44.3)	.266
Male	107 (47.4)	124(52.8)	
Ethnicity[c]			
Mandinka	93 (41.2)	106(45.1)	.490
Jola	44 (19.5)	36(15.3)	
Wollof	26 (11.5)	23(9.8)	
Fula	37 (16.4)	38(16.2)	
Others	24 (10.6)	31(13.2)	
Season			
Dry	178 (78.8)	188(80.0)	.909
Wet	48 (21.2)	47(20.0)	
Child age at follow-up visit, mean (SD)	12.24 (0.6)	12.27(0.6)	.486

Data are presented as No. (%) unless otherwise indicated.

Abbreviation: SD, standard deviation.

[a]Three missing data in the azithromycin arm.

[b]Nine missing data (2 in the azithromycin arm and 7 in the placebo arm).

[c]Three missing data (2 in the azithromycin arm and 1 in the placebo arm).

except for 1 isolate in the azithromycin arm that was susceptible to only 2 antibiotics (Figure 1).

Staphylococcus aureus

Overall, 13 of the 99 *S. aureus* isolates were resistant to azithromycin, and prevalence was similar between study arms (3.1% vs 2.6% in the azithromycin and placebo arms, respectively; OR, 1.22 [95% CI, .35–4.47]). Prevalence of *S. aureus* resis-tance against the other antibiotics was low (<3.0%) and similar between arms (Table 4). All *S. aureus* isolates from both arms were susceptible to at least 1 of the antibiotics tested (Figure 2).

Association of Azithromycin Resistance up to 28 Days and 1 Year Posttreatment (CSS)

There were 427 participants with complete information on *S. pneumoniae* and *S. aureus* resistance (samples at the 5 time points during the neonatal period and at 1 year posttreatment). For *S. pneumoniae*, 0.73% of the participants not carrying a resistance strain during the neonatal period had a resistant strain 1 year after birth; prevalence of resistant strains was sig-nificantly higher (14.3%) in those carrying a resistant strain during the neonatal period ($P < .001$). For *S. aureus*, prevalence of resistant strains at 1 year was not associated with carriage during the neonatal period (2.6% vs 3.9%; $P = .524$).

DISCUSSION

Twelve months after administering azithromycin to women in labor, the prevalence of *S. aureus* azithromycin resistance previously observed during the neonatal period had returned to baseline levels, with no differences between study arms. This was also true for *S. pneumoniae* azithromycin resistance.

Table 3. **Univariate Analysis of Bacterial Carriage by Arm After 12 Months**

Bacteria	Azithromycin (n = 226), No. (%)	Placebo (n = 235), No. (%)	OR	(95% CI)	*P* Value
Streptococcus pneumoniae					
Yes	192 (85.0)	193 (82.1)	1.23	(.73–2.08)	.413
No	34 (15.0)	42 (17.9)			
Staphylococcus aureus					
Yes	49 (21.7)	50 (21.3)	1.02	(.64–1.64)	.916
No	177 (78.3)	185 (78.7)			

Abbreviations: CI, confidence interval; OR, odds ratio.

Table 4. **Prevalence of Antibiotic-Resistant Bacteria, by Arm, After 12 Months**

Bacteria and Antibiotic		Azithromycin (n = 226), No. (%)	Placebo (n = 235), No. (%)	OR	(95% CI)	*P* Value
Streptococcus pneumoniae						
Azithromycin^{R}	Yes	4 (1.8)	2 (0.9)	2.10	(.30–23.38)	.384
	No	222 (98.2)	233 (99.1)			
Erythromycin^{R}	Yes	5 (2.2)	3 (1.3)	1.75	(.34–11.38)	.442
	No	221 (97.8)	232 (98.7)			
Chloramphenicol^{R}	Yes	8 (3.5)	3 (1.3)	2.84	(.67–16.78)	.111
	No	218 (96.5)	232 (98.7)			
Penicillin^{R}	Yes	0 (0.0)	0 (0.0)	NA		
	No	226 (100.0)	235 (100.0)			
Clindamycin^{R}	Yes	1 (0.4)	0 (0.0)	NA		.307
	No	225 (99.6)	235 (100.0)			
Cotrimoxazole^{R,a}	Yes	140 (92.1)	152 (93.3)	0.84	(.33–2.17)	.696
	No	12 (7.9)	11 (6.8)			
Staphylococcus aureus						
Azithromycin^{R,b}	Yes	7 (3.1)	6 (2.6)	1.22	(.35–4.47)	.724
	No	219 (96.9)	229 (97.5)			
Erythromycin^{R,b}	Yes	5 (2.2)	5 (2.1)	1.04	(.24–4.59)	.950
	No	221 (97.8)	230 (97.9)			
Cefoxitin^{R}	Yes	0 (0.0)	2 (0.9)	NA	(.00–1.99)	.165
	No	226 (100.0)	233 (99.2)			
Chloramphenicol^{R}	Yes	1 (0.4)	3 (1.3)	0.34	(.01–4.32)	.334
	No	225 (99.6)	232 (98.7)			
Clindamycin^{R}	Yes	6 (2.6)	7 (3.0)	0.89	(.24–3.14)	.834
	No	220 (97.4)	228 (97.0)			

Testing was done with Etest and VITEK-2.

Abbreviations: CI, confidence interval; NA, not applicable; OR, odds ratio; R, resistant.

[a]Only 63.3% (57/90) of cotrimoxazole-resistant *S. pneumoniae* isolates by disk diffusion were confirmed by Etest.

[b]Isolates of intermediate azithromycin (4 µg/mL) or erythromycin (1–4 µg/mL) resistance were considered sensitive.

Prevalence of azithromycin-resistant pneumococcal carriage was low among infants from both groups. This was expected as prevalence of resistance was low and similar between trial arms during the 4 weeks following the intervention [20]. Our results are similar to those of the Gambian trachoma trial in which chil-dren received an annual dose of azithromycin for 1 or 3 years [9] and prevalence of *S. pneumoniae* azithromycin resistance was similar between arms 6 months after dose 3. They are also consistent with the trial conducted in northern Tanzania, where no association of prevalence of macrolide-resistant *S. pneumoniae* was found 6 months following MDA-Z [25]. Nevertheless, in some studies azithromycin resistance persists after MDA-Z. In

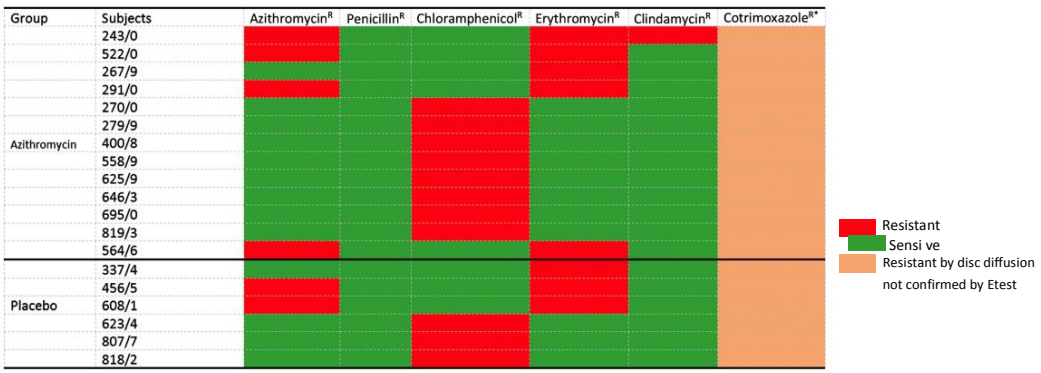


Figure 1. Heat map showing pattern of *Streptococcus pneumoniae* isolates resistant (R) to at least 1 antibiotic (except cotrimoxazole), by arm. Resistance Etest cutoff values: azithromycin, ≥2 µg/mL; penicillin, ≥8 µg/mL; chloramphenicol, ≥8 µg/mL; erythromycin, ≥1 µg/mL; and clindamycin, ≥1 µg/mL. *Cotrimoxazole susceptibility patterns for isolates resistant to at least 1 of azithromycin, penicillin, chloramphenicol, erythromycin, and clindamycin are included in the table. Resistance cutoff value was ≤18 mm using disk diffusion.

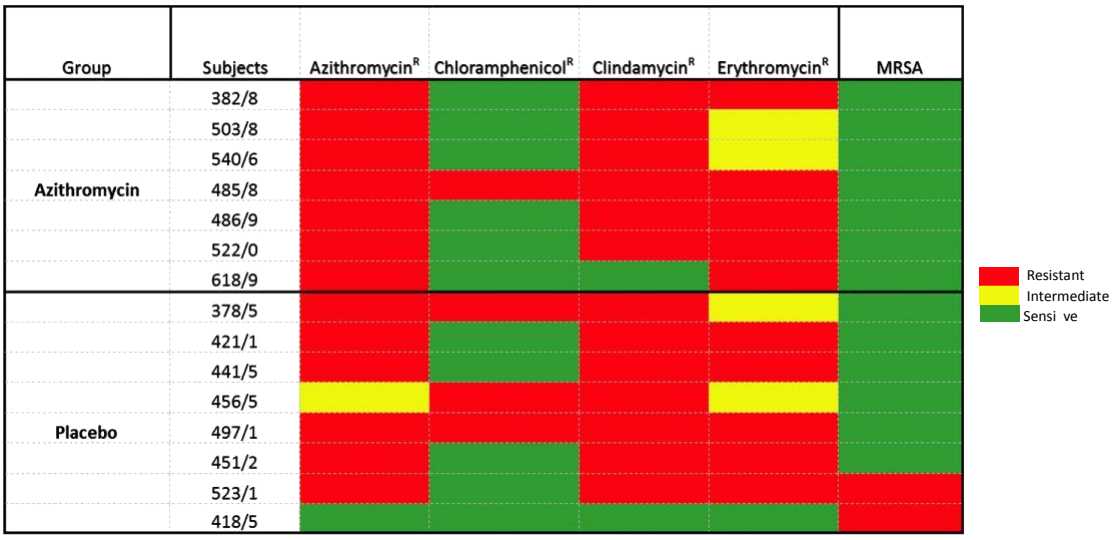


Figure 2. Heat map showing pattern of *Staphylococcus aureus* isolates resistant (R) to at least 1 antibiotic, by arm. Resistance Etest cutoff values: azithromycin, ≥8 µg/ mL; chloramphenicol, ≥32 µg/mL; erythromycin, ≥16 µg/mL; clindamycin, ≥4 µg/mL; cefoxitin for methicillin-resistant *Staphylococcus aureus* (MRSA), ≥8. Intermediate Etest cutoff values: azithromycin, 4 µg/mL; erythromycin, 1–4 µg/mL.

Ethiopia, prevalence of azithromycin-resistant *S. pneumoniae* increased after 4 rounds of MDA-Z for trachoma control and did not return to baseline 12 months after the last round [14]. This is probably due to the higher prevalence of resistance at baseline (9.2%) [14] as compared to The Gambia (1.4%) [20] and Tanzania (0%) [25], and to the larger number of MDA-Z rounds. Indeed, frequent exposure to treatment and higher resistance at baseline are associated with increased prevalence of macrolide resistance that persists for a very long time [26].

Only 1 isolate (from the azithromycin arm) showed resistance to both macrolides and clindamycin, suggesting constitutive resistance. All clindamycin-sensitive, macrolide-resistant isolates had a negative D-test, indicating no evidence of induc-ible resistance. Macrolide exposure could potentially induce clindamycin resistance through methylation of the common ribosomal binding site for macrolides, clindamycin as well as streptogramin B (MLS_B), often referred to as the MLS_B pheno-type [27]. However, in our study population, macrolide expo-sure did not induce resistance to clindamycin.

For cotrimoxazole, two-thirds of the *S. pneumoniae* isolates resistant by disk diffusion were confirmed by Etest and, there-fore, actual resistance was probably around 60%. Such high prevalence among the children has been previously described [28] and is probably the result of high cotrimoxazole use in The Gambia; no increase as a result of the intervention was observed.

Nearly all *S. aureus* isolates resistant to azithromycin were resistant to erythromycin and approximately two-thirds were also resistant to clindamycin, suggesting that the underlying mechanism was constitutive, mediated by the *erm* gene [27]. There were 2 cases of methicillin-resistant *S. aureus* (MRSA) in the study, both in children from the placebo arm, and therefore not associated with the intervention. To our knowledge, this is the first time MRSA has been reported from carriage in The Gambia, although it was previously reported from invasive isolates [29].

This study had some limitations. One is the lack of information between the last sample collection in the main trial and the survey done at the infants' first birthday. We were unable to determine how long *S. aureus*–resistant isolates persisted. Such information is important to determine the potential risk of resistance transmission and establishment within the population. Despite the lack of information on antibiotic use by the study participants between 2 and 12 months of age, we did not expect major differences between trial arms, as this was a randomized trial. The survey carried out at about 12 months postintervention was able to include about two-thirds of study participants, which is a substantial proportion of the study population; we did not observe significant differences between children included in the survey and those left out. Exposure to a macrolide in our study population may have also resulted in the emergence of macrolide-resistant, gram-negative bacteria. In Tanzania, rectal swabs collected from young children following MDA-Z exposure were significantly associated with higher azithromycin-resistant *Escherichia coli* carriage at 1 month post-MDA (OR, 15.27; $P < .001$) and all subsequent surveys [30]. Our analysis was based on gram-positive bacteria as we only collected NPS.

In conclusion, administering 2 g of azithromycin to Gambian women in labor induced a transient azithromycin resistance in *S. aureus* that lasted <12 months. Although the long-term impact on prevalence of resistance of the 2 bacteria is reassur-ing, pathogenicity and transmissibility of resistant *S. aureus* strains observed in the short term warrant further investigation.

Notes

Acknowledgments. The authors give special thanks to the field team for assisting with sample collection, and to the study participants for agreeing to take part in the study.

Financial support. This work was supported by the Medical Research Council (MRC) Unit The Gambia PhD fellowship. The main trial was jointly funded by the UK MRC and the UK Department for International Development (DFID) under the MRC/DFID Concordat agreement (reference number MR/J010391/1) and is also part of the EDCTP2 program supported by the European Union.

Potential conflicts of interest. All authors: No reported conflicts of Interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Parnham MJ, Erakovic Haber V, Giamarellos-Bourboulis EJ, Perletti G, Verleden GM, Vos R. Azithromycin: mechanisms of action and their relevance for clinical applications. Pharmacol Ther **2014**; 143:225–45.
2. Bignell C, Garley J. Azithromycin in the treatment of infection with *Neisseria gon-orrhoeae*. Sex Transm Infect **2010**; 86:422–6.
3. Harding-Esch EM, Sillah A, Edwards T, et al; Partnership for Rapid Elimination of Trachoma (PRET) Study Group. Mass treatment with azithromycin for trachoma: when is one round enough? Results from the PRET Trial in the Gambia. PLoS Negl Trop Dis **2013**; 7:e2115.
4. Yohannan J, Munoz B, Mkocha H, et al. Can we stop mass drug administration prior to 3 annual rounds in communities with low prevalence of trachoma? PRET Ziada trial results. JAMA Ophthalmol **2013**; 131:431–6.
5. Gebre T, Ayele B, Zerihun M, et al. Comparison of annual versus twice-yearly mass azithromycin treatment for hyperendemic trachoma in Ethiopia: a clus-ter-randomised trial. Lancet **2012**; 379:143–51.
6. Schémann JF, Guinot C, Traore L, et al. Longitudinal evaluation of three azithromycin distribution strategies for treatment of trachoma in a sub-Saharan African country, Mali. Acta Trop **2007**; 101:40–53.
7. Lee JS, Muñoz BE, Mkocha H, Gaydos CA, Quinn TC, West SK. The effect of multiple rounds of mass drug administration on the association between ocular *Chlamydia trachomatis* infection and follicular trachoma in preschool-aged children. PLoS Negl Trop Dis **2014**; 8:e2761.
8. Biebesheimer JB, House J, Hong KC, et al. Complete local elimination of infectious trachoma from severely affected communities after six biannual mass azithromycin distributions. Ophthalmology **2009**; 116:2047–50.
9. Burr SE, Milne S, Jafali J, et al. Mass administration of azithromycin and *Streptococcus pneumoniae* carriage: cross-sectional surveys in the Gambia. Bull World Health Organ **2014**; 92:490–8.
10. Porco TC, Gebre T, Ayele B, et al. Effect of mass distribution of azithromycin for trachoma control on overall mortality in Ethiopian children: a randomized trial. JAMA **2009**; 302:962–8.
11. Luntamo M, Kulmala T, Cheung YB, Maleta K, Ashorn P. The effect of antenatal monthly sulphadoxine-pyrimethamine, alone or with azithromycin, on foetal and neonatal growth faltering in Malawi: a randomised controlled trial. Trop Med Int Health **2013**; 18:386–97.
12. Unger HW, Ome-Kaius M, Wangnapi RA, et al. Sulphadoxine-pyrimethamine plus azithromycin for the prevention of low birthweight in Papua New Guinea: a randomised controlled trial. BMC Med **2015**; 13:9.
13. Coles CL, Mabula K, Seidman JC, et al. Mass distribution of azithromycin for trachoma control is associated with increased risk of azithromycin-resistant *Streptococcus pneumoniae* carriage in young children 6 months after treatment. Clin Infect Dis **2013**; 56:1519–26.
14. Skalet AH, Cevallos V, Ayele B, et al. Antibiotic selection pressure and macrolide resistance in nasopharyngeal *Streptococcus pneumoniae*: a cluster-randomized clinical trial. PLoS Med **2010**; 7:e1000377.
15. Haug S, Lakew T, Habtemariam G, et al. The decline of pneumococcal resistance after cessation of mass antibiotic distributions for trachoma. Clin Infect Dis **2010**; 51:571–4.
16. Bojang E, Jafali J, Perreten V, et al. Short-term increase in prevalence of naso-pharyngeal carriage of macrolide-resistant *Staphylococcus aureus* following mass drug administration with azithromycin for trachoma control. BMC Microbiol **2017**; 17:75.
17. Unger HW, Aho C, Ome-Kaius M, et al. Impact of intermittent preventive treatment in pregnancy with azithromycin-containing regimens on maternal nasopharyngeal carriage and antibiotic sensitivity of *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Staphylococcus aureus*: a cross-sectional survey at delivery. J Clin Microbiol **2015**; 53:1317–23.
18. Oluwalana C, Camara B, Bottomley C, et al. Azithromycin in labor lowers clinical infections in mothers and newborns: a double-blind trial. Pediatrics **2017**; 139. doi:10.1542/peds.2016-2281.
19. Burr SE, Camara B, Oluwalana C, et al. Does azithromycin given to women in labour decrease ocular bacterial infection in neonates? A double-blind, random-ized trial. BMC Infect Dis **2017**; 17:799.
20. Roca A, Oluwalana C, Bojang A, et al. Oral azithromycin given during labour decreases bacterial carriage in the mothers and their offspring: a double-blind randomized trial. Clin Microbiol Infect **2016**; 22:565.e1–9.
21. Roca A, Oluwalana C, Camara B, et al. Prevention of bacterial infections in the newborn by pre-delivery administration of azithromycin: study protocol of a randomized efficacy trial. BMC Pregnancy Childbirth **2015**; 15:302.
22. O'Brien KL, Nohynek H; World Health Organization Pneumococcal Vaccine Trials Carriage Working Group. Report from a WHO Working Group: standard method for detecting upper respiratory carriage of *Streptococcus pneumonia*e. Pediatr Infect Dis J **2003**; 22:e1–11.
23. Clinical Laboratory and Standards Institute. Performance standards for antimicrobial susceptibility testing. 27th ed. Wayne, PA: CLSI, **2017**:224.
24. Steward CD, Raney PM, Morrell AK, et al. Testing for induction of clindamycin resistance in erythromycin-resistant isolates of *Staphylococcus aureus*. J Clin Microbiol **2005**; 43:1716–21.
25. Batt SL, Charalambous BM, Solomon AW, et al. Impact of azithromycin administration for trachoma control on the carriage of antibiotic-resistant *Streptococcus pneumoniae*. Antimicrob Agents Chemother **2003**; 47:2765–9.
26. Ho DK, Sawicki C, Grassly N. Antibiotic resistance in *Streptococcus pneumoniae* after azithromycin distribution for trachoma. J Trop Med **2015**; 2015:917370.
27. Leclercq R. Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implications. Clin Infect Dis **2002**; 34:482–92.
28. Hill PC, Onyeama CO, Ikumapayi UN, et al. Bacteraemia in patients admitted to an urban hospital in West Africa. BMC Infect Dis **2007**; 7:2.
29. Senghore M, Bayliss SC, Kwambana-Adams BA, et al. Transmission of *Staphylococcus aureus* from humans to green monkeys in The Gambia as revealed by whole-genome sequencing. Appl Environ Microbiol **2016**; 82:5910–7.
30. Seidman JC, Coles CL, Silbergeld EK, et al. Increased carriage of macrolide-resis-tant fecal *E. coli* following mass distribution of azithromycin for trachoma con-trol. Int J Epidemiol **2014**; 43:1105–13.

CHAPTER 5

5.1 PhD Manuscript #2

5.2 GENOMIC INVESTIGATION OF *STAPHYLOCOCCUS AUREUS* RECOVERED FROM GAMBIAN WOMEN AND NEWBORNS FOLLOWING AN ORAL DOSE OF INTRAPARTUM AZITHROMYCIN

Published 19th August 2019 in ***Journal of antimicrobial chemotherapy***

J Antimicrob Chemother 2019; 74: 3170–3178
doi:10.1093/jac/dkz341 Advance Access publication 19 August 2019

Journal of Antimicrobial Chemotherapy

Genomic investigation of Staphylococcus aureus recovered from Gambian women and newborns following an oral dose of intra-partum azithromycin

Abdoulie Bojang[1], Sarah L. Baines[2], Liam Donovan[2], Romain Guerillot[2], Kerrie Stevens[3], Charlie Higgs[3], Christian Bottomley[4], Ousman Secka[2], Mark B. Schultz[3], Anders Gonc¸alves da Silva[3], Torsten Seemann[2], Timothy P. Stinear[2], Anna Roca[1] and Benjamin P. Howden[2,3]*

[1]Medical Research Council Unit The Gambia at the London School of Hygiene and Tropical Medicine, Fajara, The Gambia; [2]Doherty Applied Microbial Genomics, Department of Microbiology & Immunology, The University of Melbourne at The Peter Doherty Institute for Infection & Immunity, Melbourne, Victoria, Australia; [3]Microbiological Diagnostic Unit Public Health Laboratory, Department of Microbiology & Immunology, The University of Melbourne at The Peter Doherty Institute for Infection & Immunity, Melbourne, Victoria, Australia; [4]Medical Research Council Tropical Epidemiology Group, London School of Hygiene and Tropical Medicine, London, UK

*Corresponding author. E-mail: bhowden@unimelb.edu.au

Received 21 February 2019; returned 20 May 2019; revised 2 July 2019; accepted 9 July 2019

Background: Oral azithromycin given during labour reduces carriage of bacteria responsible for neonatal sepsis, including Staphylococcus aureus. However, there is concern that this may promote drug resistance.

Objectives: Here, we combine genomic and epidemiological data on S. aureus isolated from mothers and babies in a randomized intra-partum azithromycin trial (PregnAnZI) to describe bacterial population dynamics and resistance mechanisms.

Methods: Participants from both arms of the trial, who carried S. aureus in day 3 and day 28 samples post-intervention, were included. Sixty-six S. aureus isolates (from 7 mothers and 10 babies) underwent comparative genome analyses and the data were then combined with epidemiological data. Trial registration (main trial): ClinicalTrials.gov Identifier NCT01800942.

Results: Seven S. aureus STs were identified, with ST5 dominant (n=40, 61.0%), followed by ST15 (n=11, 17.0%). ST5 predominated in the placebo arm (73.0% versus 49.0%, P=0.039) and ST15 in the azithromycin arm (27.0% versus 6.0%, P=0.022). In azithromycin-resistant isolates, msr(A) was the main macrolide resistance gene (n=36, 80%). Ten study participants, from both trial arms, acquired azithromycin-resistant S. aureus after initially har-bouring a susceptible isolate. In nine (90%) of these cases, the acquired clone was an msr(A)-containing ST5 S. aureus. Long-read sequencing demonstrated that in ST5, msr(A) was found on an MDR plasmid.

Conclusions: Our data reveal in this Gambian population the presence of a dominant clone of S. aureus harbouring plasmid-encoded azithromycin resistance, which was acquired by participants in both arms of the study. Understanding these resistance dynamics is crucial to defining the public health drug resistance impacts of azithromycin prophylaxis given during labour in Africa.

Introduction

Azithromycin, a second-generation broad-spectrum macrolide, is used to treat infections such as pneumonia, middle ear infections and sexually transmitted infections.[1,2] It has also been used in mass drug administration (MDA) campaigns to control trachoma in several endemic countries in Africa.[3–5] The impact of these MDA campaigns has varied from one country to another.[6–8] MDA with azithromycin (MDA-Z) may have beneficial effects beyond trachoma control, having been shown to reduce asymptomatic pneumococcal carriage for at least 1 month,[9] and all-cause mortality in children.[10,11] However, a concern is that MDA-Z has been associated with an increase in the prevalence of macrolide-resistant bacterial species, even after the administration of only a single dose.[12] The spread of these resistant bacterial populations and the associated risk to regions that may implement MDA-Z are not fully understood.

Staphylococcus aureus is regularly implicated as a species in which azithromycin resistance emerges following MDA-Z campaigns. In Papua New Guinea, the proportion of azithromycin-resistant (azithromycin^R) S. aureus was five times higher among pregnant women treated with azithromycin than in those in the control group.[13] A study in rural Gambia showed that three annual rounds of MDA-Z were associated with a long-term increase in the prevalence of azithromycin^R S. aureus. There are three recognized types of acquired macrolide resistance mechanisms in S. aureus: (i) methylation of the ribosomal target (erm gene);[14,15] (ii) active efflux [msr(A) gene];[14,16,17] and (iii) inactivation of the macrolide (mph/ere gene).[18,19] In the absence of these genes, mutations in ribosomal proteins have been implicated in macrolide resistance.[20] As azithromycin is a synthetic analogue of erythromycin, it is presumed that these resistance mechanisms are active against both antimicrobials. However, molecular data confirming this association are limited for azithromycin, in particular for the efflux mechanism encoded by msr(A). Further, there are limited data available on the distribution of these macrolide resistance-encoding genes following public health interventions using azithromycin.

A recent MDA-Z trial in The Gambia, the Prevention of Bacterial Infections in Newborn (PregnAnZI) trial, a double-blinded placebo-controlled trial in which oral intra-partum azithromycin (2 g) was administered, showed that phenotypic resistance to azithromycin was associated with S. aureus isolates harbouring msr(A) or erm(C) genes.[21] Public health interventions using antibiotics, in addition to driving the emergence of resistance, may also greatly alter the local molecular epidemiology of S. aureus. The distribution of dom-inant MSSA clones in Africa is heterogeneous,[22] and in The Gambia there is currently a paucity of data on the prevalence of S. aureus STs, although ST15 and ST5 have been most commonly reported.[23,24] The ST15 lineage in Africa has been reported to fre-quently harbour genes encoding the Panton–Valentine leucocidin (PVL) toxin (25.9%–90.0%) and enterotoxin A (22.0%–74.6%), sug-gesting a potential for increased virulence in this lineage.[22]

The recent PregnAnZI trial was undertaken to assess the efficacy of one oral dose of azithromycin administered to women during labour in lowering bacterial carriage both in the mother and in her newborn as a necessary step to reduce puerperal and neonatal sepsis. The trial revealed that azithromycin treatment significantly decreased carriage of S. aureus, group B Streptococcus and Streptococcus pneumoniae, but increased the prevalence of azithromycin^R S. aureus, amongst the population of bacterial isolates recovered during a 28 day follow-up period.[12] However, carriage of the latter was observed to wane in babies 12 months after delivery.[25]

The primary aims of this study were to: (i) use genomics to characterize the population of azithromycin^R S. aureus recovered from mothers and babies during a 28 day follow-up period; (ii) identify the genetic mechanisms responsible for azithromycin resistance in this population and their genetic context; and (iii) explore the potential roles of clonal replacement and transmission of azithromycin^R S. aureus at an individual patient level.

Patients and methods

Additional Materials and methods are provided in the Supplementary data, available at JAC Online.

PregnAnZI trial

The PregnAnZI trial was a Phase III, double-blind, placebo-controlled trial where 829 pregnant women attending the labour ward received a single oral dose (2 g) of azithromycin or placebo (ratio 1:1). The study protocol has been described elsewhere.[26] Participants were monitored for 8 weeks and nasopharyngeal swabs were collected during the first 4 weeks of the follow-up (day 0 for mothers and days 3, 6, 14 and 28 for mothers and babies).

Trial registration (main trial): ClinicalTrials.gov Identifier NCT01800942.

Ethics

The trial was approved by the Joint MRC/Gambia Government Ethics Committee. Mothers of children signed informed consent.

Sample selection

To explore potential genetic diversity amongst azithromycin^R S. aureus, we stratified isolates collected at day 3 and day 28 into four groups (Figure 1). Two groups were participants in the azithromycin treatment arm where an azithromycin^R S. aureus was recovered at both timepoints (group 1) or only at day 28, with an azithromycin-susceptible (azithromycin^S) S. aureus iden-tified at day 3 (group 3). The other two groups represented the same micro-biological division, but for samples recovered from participants assigned to the placebo arm [azithromycin^R S. aureus at day 3 and day 28 (group 2) or azithromycin^S S. aureus at day 3 and azithromycin^R S. aureus at day 28 (group 4)]. A total of 17 participants (34 isolates) were selected from these four groups. In addition, all S. aureus recovered from the above subjects at other timepoints (days 0, 6 and 14) were included.

WGS and bioinformatic analyses

WGS was performed on the NextSeq 500 (Illumina) using 2%150 bp chemistry. One isolate (S80062MN28) was subjected to long-read sequencing on the RS-II (Pacific Biosciences). Bioinformatic approaches and analyses included assembly, annotation and comparative genomics. The global S. aureus phylogeny was inferred using publicly available genomes. Antibiotic resistance and virulence gene detection was performed using the NCBI antimicrobial resistance database (ncbi: updated 20 September 2018) and the virulence factor database (vfdb: updated 14 August 2018). All se-quence data generated for this study have been made publicly available through the European Nucleotide Archive, project accession PRJEB31151.

Cloning and transformation of erm(C) and msr(A) genes

An empty vector control and a vector containing erm(C) or msr(A) were electroporated into an ST5 azithromycin^S S. aureus strain (S70065MN00). Colonies were screened to confirm the presence of either erm(C) or msr(A) genes using PCR.

Bacterial conjugation

Duplicate conjugation experiments were performed using an azithromycin-resistant donor strain (S80062MN28) and an azithromycin-susceptible re-cipient strain (S70065MN00). Resistance profiles of donor, recipient and transconjugants are described in Table S1.

Antimicrobial susceptibility testing

Phenotypic susceptibility to azithromycin and erythromycin was determined using Etest (bioMe´rieux), performed as per the manufacturer's recommendations.

Statistics

Fisher's exact test was used to compare the prevalence of STs or macrolide resistance genes between the azithromycin and placebo groups. A P value

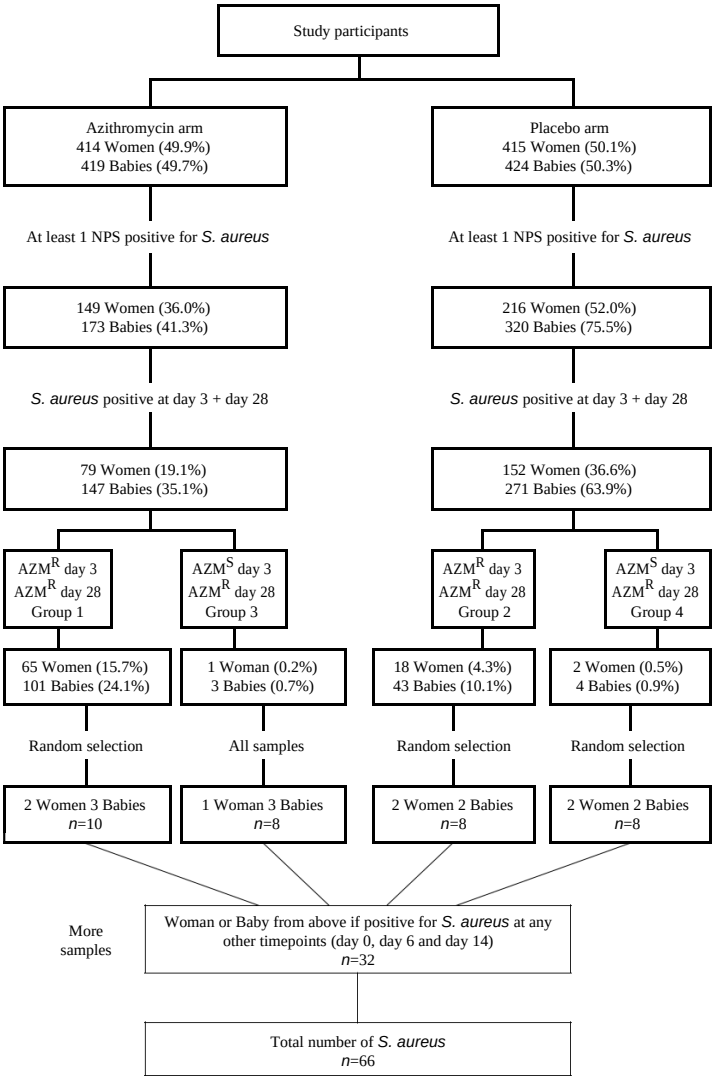


Figure 1. Flow chart showing the sample selection criteria from women and babies in the PregnAnZI trial. NPS, nasopharyngeal swab; AZM^R, azithro-mycin resistant; AZM^S, azithromycin susceptible. Resistance Etest cut-off values: azithromycin 8 mg/L.

of 0.05 was used as the cut-off for statistical significance. All analyses were done using STATA/SE v12.1 (https://www.stata.com/).

Results and discussion

Genomic characterization of the study population

A total of 66 S. aureus isolates recovered from 7 mothers and 10 babies were included in this study. Of the 66 isolates, half (n=33) were recovered from the nine participants selected from the azithromycin-treatment arm and the other half from the eight participants selected from the placebo arm of the PregnAnZI trial.

In silico MLST of the 66 S. aureus isolates revealed seven different STs: ST1 [and a novel ST1 single-locus variant (ST1-SLV)], ST5, ST8, ST15, ST152 and ST669 (Table 1). Overall ST5 was the dominant ST, representing 61.0% of isolates (n=40) recovered from 14 partic-ipants, followed by ST15, representing 17.0% of isolates (n=11) recovered from 7 participants. The prevalence of ST5 was higher in the placebo arm (73.0% versus 49.0%, P=0.039), while the preva-lence of ST15 was higher in the azithromycin treatment arm (27.0% versus 6.0%, P=0.022). These finding are largely consistent with previous molecular epidemiological data from The Gambia, in which ST15 and ST5 were common STs identified in cases of both

Table 1. Distribution of STs and macrolide resistance amongst the study population

	All samples		Azithromycin treatment arm		Placebo treatment arm	
MLST	all	AZM^R	all	AZM^R	all	AZM^R
ST5	14 (40)	14 (36)	6 (16)	6 (16)	8 (24)	8 (20)
ST15	7 (11)	1 (1)	5 (9)	1 (1)	2 (2)	0 (0)
ST1	1 (4)	0 (0)	–	–	1 (4)	0 (0)
ST669	1 (4)	1 (4)	1 (4)	1 (4)	–	–
ST8	1 (3)	0 (0)	–	–	1 (3)	0 (0)
ST152	1 (3)	1 (3)	1 (3)	1 (3)	–	–
ST1-SLV	1 (1)	1 (1)	1 (1)	1 (1)	–	–

AZM^R, azithromycin resistant.
Data are shown as number of participants (number of isolates).

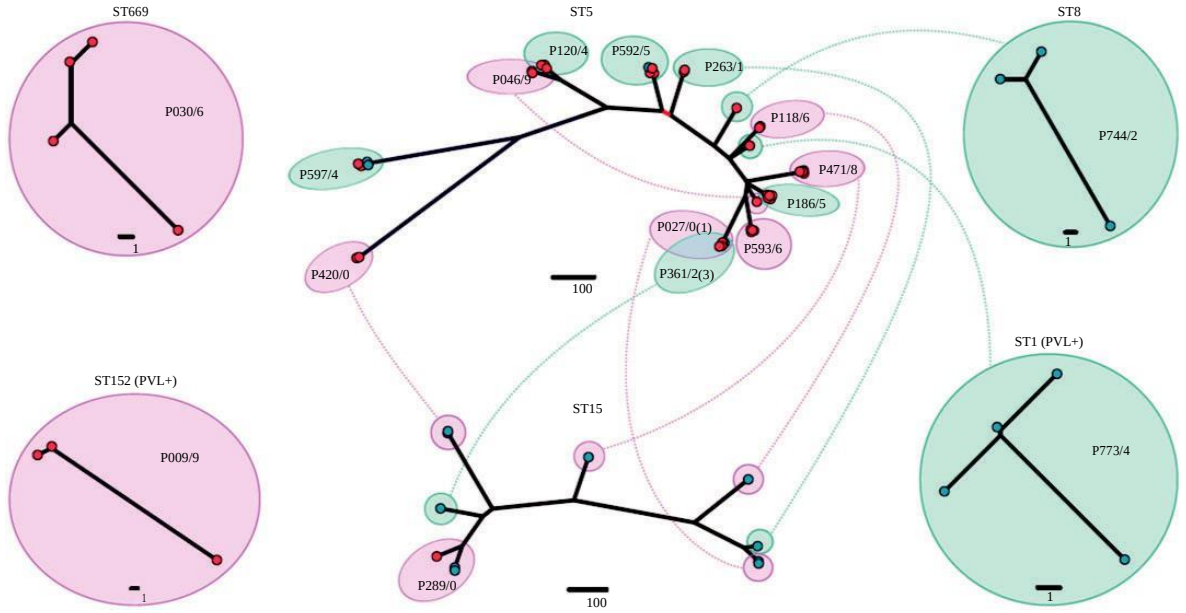


Figure 2. Distribution and phylogenetic relatedness of participant isolates. Illustrated are ML phylogenetic trees for each ST identified in the study population (excluding the ST1-SLV), inferred from whole-genome alignments. Branch tips are coloured based on phenotypic susceptibility to azithromycin (blue=susceptible and red=resistant, as determined by Etest). Scale bar indicates the estimated substitutions per site. Isolates recovered from the same participant are indicated by a larger circle, labelled with the participant ID and coloured based on the treatment arm to which the partici-pant was assigned (purple=azithromycin and green=placebo). Participants with isolates representing multi-STs are connected by a dotted line. Isolates belonging to ST1 and ST152 were found to harbour the PVL toxin-encoding gene, lukFS.

colonization and disease.[23,24] Unlike previous reports of increasing PVL positivity in the Gambian ST15 population,[22] the lukFS genes were only identified in the isolates representing ST1 and ST152 (Figure 2).

To explore the genetic relationships between isolates, a max-imum likelihood (ML) phylogenetic tree was inferred for each ST (excluding the ST1-SLV). In the case of ST1, ST8, ST152 and ST669, each clone was recovered from a single participant and demon-strated limited core SNP diversity (Figure 2). This finding suggested that these participants maintained the same S. aureus clone over the 28 day follow-up period. Only ST5 and ST15 were recovered from multiple participants (n=14 and 7, respectively) and as a group demonstrated core SNP diversity. Isolates of the same ST recovered from the same participant, however, largely formed a single, genetically distinct clade within both phylogenetic trees (Figure 2). The median pairwise core SNP distance between isolates recovered from the same participant was 2.5 (±16.4, range 0–85) for ST5 and 104 (±61.2, range 0–125) for ST15; for isolates

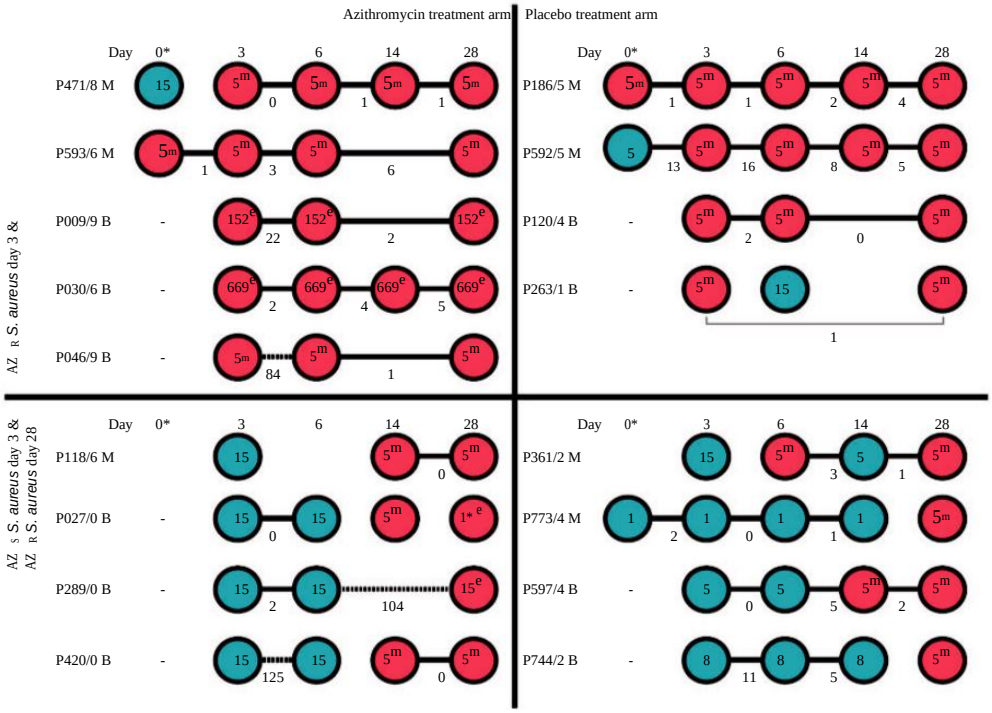


Figure 3. Individual participant timelines. Illustrated are the isolate timelines for each participant selected for the study, grouped based on the selection criteria outlined in Figure 1. Each participant is indicated by their participant ID and the adjacent circles represent the isolates recovered from samples taken prior to the intervention (day 0 for mothers) and following the intervention on days 3, 6, 14 and 28 (for mothers and babies). Isolate circles are coloured based on the phenotypic susceptibility to azithromycin (blue=susceptible and red=resistant, as determined by Etest). The internal number indicates the in silico MLST of the isolate and the superscript letter indicates the macrolide resistance gene detected, 'm' for msr(A) and 'e' for erm(C). Black lines connecting adjacent isolates indicate that the same clone has probably been maintained, with the pairwise core SNP distance provided. Alternatively, gaps between adjacent isolates indicate a potential clonal replacement event. AZM^R, azithromycin resistant; AZM^S, azithromycin susceptible.

recovered from different participants it was 95 (±34.2, range 0–200) for ST5 and 115 (±24.1, range 9–136) for ST15. A potential clonal replacement event probably occurred in one participant (P046/9), with the ST5 MSSA isolate recovered on day 3 having a pairwise core SNP distance of 84 and 85 to the ST5 MSSA isolates recovered on day 6 and 28, respectively (Figure 3, Dataset S3). Another two partic-ipants (P027/0 and P0361/2) appeared to be involved in a potential transmission event as the four ST5 MSSA isolates recovered from these participants demonstrated a pairwise core SNP distance of 3 (Dataset S3). There was no apparent clustering of participant iso-lates based on the treatment received (Figure 2).

Genetic basis of azithromycin resistance

Of the 66 S. aureus isolates, 21 were phenotypically $azithromycin^S$ and 45 $azithromycin^R$ as determined by Etest. None of the $azithromycin^S$ S. aureus carried a known azithromycin resistance-conferring gene, whereas all $azithromycin^R$ S. aureus were found to carry either an erm(C) (n=9, 20%) or msr(A) (n=36, 80%) gene (Figure 3), genes associated with macrolide resistance. Mutations in genes encoding ribosomal proteins (rplD, rplV and 23S rRNA) were investigated. Only two missense mutations were identified: RplD $A_{133}D$ and RplD $T_{145}I$. Neither has been previously reported, and they were identified in four isolates carrying erm(C) (P471/8) and one isolate carrying msr(A) (P027/0), respectively.

The two macrolide resistance genes are associated with different phenotypes: carriage of msr(A) among staphylococci is associated with phenotypic resistance to 14-membered (clarithromycin, dirith-romycin and erythromycin) or 15-membered (azithromycin) ring macrolides and streptogramin A, but susceptibility to 16-membered ring macrolides.[18] Carriage of erm genes in staphylococci is associ-ated with a broader phenotypic resistance depending on whether the gene is inducible or constitutively expressed. The former, often referred to as $iMLS_B$, mediates resistance to 14- and 15-membered macrolides and streptogramin B, but susceptibility to 16-membered macrolides and lincosamides, with a risk of constitutive expression arising in vivo.[18,27] The latter, denoted by $cMLS_B$, mediates resistance to all macrolide, lincosamide and streptogramin B antibiotics.[18]

The prevalence of each gene differed significantly when iso-lates were grouped based on the intervention which the partici-pant received (P=0.002). The erm(C) gene was carried by isolates belonging to ST15, ST152, ST669 and the ST1-SLV, and were only

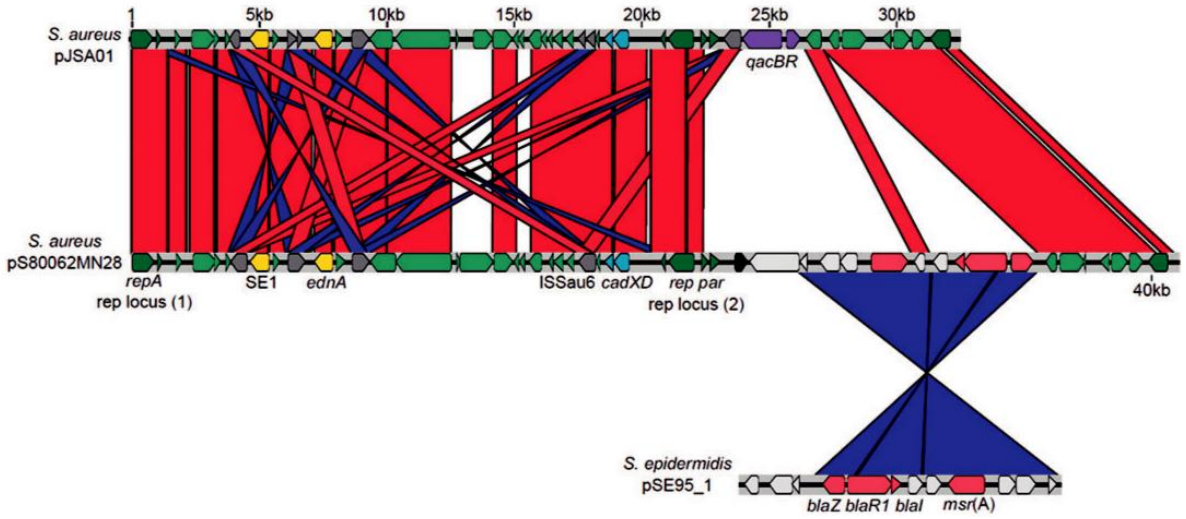


Figure 4. Schematic for the structure of pS80062MN28. Illustrated is the structure of pS80062MN28 (central sequence PRJEB31151) with genes of interest annotated. pS800262MN28 has been aligned with S. aureus plasmid pJSA01 (accession AP014922.1) and S. epidermidis plasmid pSE95_1 (ac-cession CP024438.1). Connecting lines indicate sequences that share >99% nucleotide sequence homology, coloured red or blue if the sequence was present in the same or reverse orientation, respectively.

recovered from participants assigned to the azithromycin treat-ment arm. Conversely, the msr(A) gene was exclusively associated with ST5 MSSA isolates and was recovered from participants assigned to either treatment arm (Figure 3). As both resistance genes would provide protection against azithromycin, the factors mediating the differences in the prevalence of each gene between participants assigned to the different interventions are unclear. This finding requires confirmation in a larger isolate dataset.

Genetic context of msr(A)

In the ST5 lineage identified in this study, msr(A) was found to be located on an MDR staphylococcal plasmid (Figure 4). pS80062MN28 is 41069 bp in length and demonstrates significant similarity to the published S. aureus plasmid pJSA01 (accession AP014922.1) (Figure 4).[28] Plasmid pJSA01 carries two virulence genes, a newly discovered enterotoxin (SE1) and the epidermal cell differentiation inhibitor A (EDIN-A) encoded by ednA.[28,29] Additionally, it carries genes encoding cadmium resistance (cadXD) and biocide tolerance (qacBR).[28] Plasmid pS80062MN28 was found to carry both putative virulence genes and cadXD, but not qacBR, and contained a 12 kb region not present in pJSA01 (Figure 4). Approximately 9 kb of this region demonstrated signifi-cant similarity to a putative S. epidermidis plasmid pSE95_1 (acces-sion CP024438.1),[30] and included msr(A) and the b-lactamase resistance-encoding gene blaZ (Figure 4). In all msr(A)-harbouring ST5 MSSA isolates, an 27 kb contig was identified, representing the plasmid region that spans from the ISSau6-type transposase upstream of cadXD to another copy upstream of SE1 (Figure 4), suggesting that all isolates probably carried a pS80062MN28-like plasmid.

To demonstrate that msr(A) was responsible for macrolide re-sistance in the ST5 lineage, a copy of the gene was transformed into a macrolide-susceptible ST5 isolate from the study collection (S70065MN00), using vector pRAB11.[31] Two allelic variants of erm(C) [erm(C)_2 and erm(C)_13] were also tested. Transformation of an empty vector did not result in a change in azithromycin or erythromycin susceptibility in isolate S70065MN00; however, transformation of any one of the three re-sistance genes resulted in an increase in the azithromycin MIC from 1.0 to >256 mg/L (Figure S1), the first time introduction of msr(A) or erm(C) genes in a previously azithromycin^{S} S. aureus clinical isolate has been shown to result in azithromycin resistance. All clinical S. aureus isolates carrying either the erm(C) or msr(A) gene had similarly high MIC values as those shown in the cloning experiment (MIC 256 mg/L). The MIC values for all azithromycin-resistant and -susceptible isolates are included in Dataset S1 (col-umn F). WGS of transformants demonstrated no additional muta-tions potentially contributing to azithromycin resistance. Therefore, acquisition of msr(A), probably through uptake of the plasmid, is responsible for macrolide resistance in the ST5 lineage. Results of the bacterial conjugation experiment demonstrated that the transfer of the msr(A)-containing resistance plasmid was <1.06%10$^{\#14}$ between S. aureus, indicating a low frequency of transmission.

Contextualization of the study population

To contextualize the Gambian isolates of this study with the global population of S. aureus, an ML phylogenetic tree comprising 7126 publicly available S. aureus genomes together with the sequenced isolates of this study was constructed, as well as subtrees of CC5 and CC15 (Figure 5). The subtree of CC5 isolates (Figure 5b) illus-trated that the Gambian ST5 MSSA lineage identified in this study represented a single monophyletic clade, consistent with local ex-pansion of this clone. The most closely related isolate to this clade

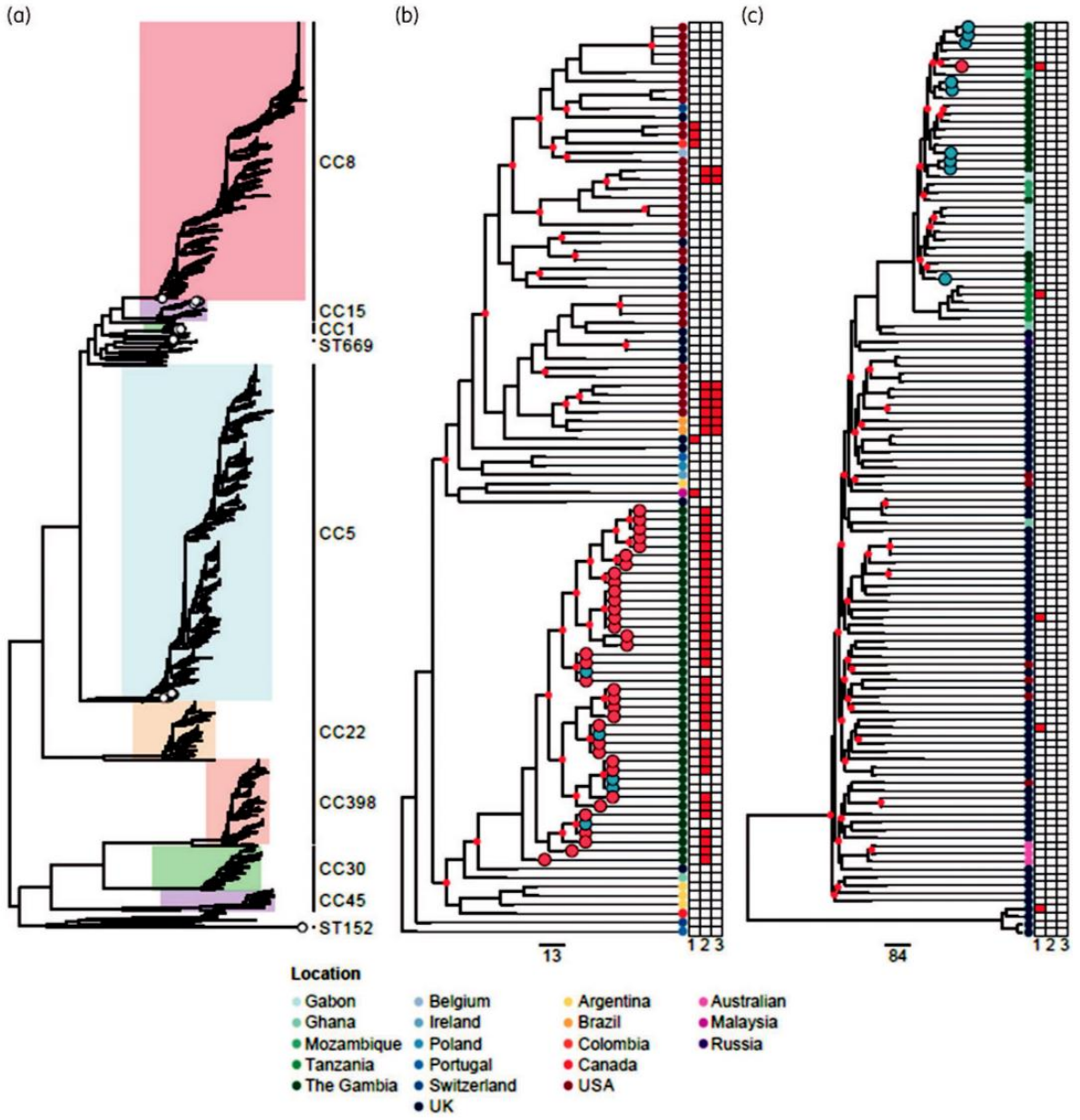


Figure 5. Phylogenetic contextualization of study isolates. (a) Illustrated is an ML phylogenetic tree for a global population of 7192 S. aureus iso-lates. The Gambian isolates identified in this study are indicated by white circles located at the branch tips. (b) A subtree of CC5 isolates and (c) a subtree of CC15 isolates. In both subtrees, branches with <85% support (approximate likelihood ratio test) are labelled with a red circle. The Gambian isolates identified in this study are indicated by circles located at the branch tips, coloured based on phenotypic susceptibility to azithro-mycin (blue=susceptible and red=resistant, as determined by Etest). Adjacent to each subtree is a vertical line of circles indicating the countries from which the isolates were recovered (refer to key); the heatmap indicates the presence or absence of three macrolide resistance genes: erm(C) (1), msr(A) (2) and mph/ere (3). The scale bar indicates the estimated number of core genome substitutions.

was recovered from the UK (435 SNPs). Given the distribution of macrolide resistance genes in this CC5 population (Figure 5b), there is no clear source for pS80062MN28 in the Gambian ST5 lineage. Therefore, it remains unclear if the MDA-Z trial promoted the expansion of this lineage (already carrying the plasmid) or provided the necessary selection pressure for plasmid uptake from a cur-rently unidentified source. The potential consequences of either evolutionary mechanism are greater than simply increasing the prevalence of azithromycin resistance in a region due to the co-location of msr(A) with other resistance and virulence genes on the plasmid. Therefore, the impact of pS80062MN28 carriage for bacterial fitness and pathogenicity requires further investigation.

Population dynamics of azithromycin^R S. aureus carriage

As all participants were sampled at multiple and consistent time-points following administration of either azithromycin or the pla-cebo, it presented an opportunity to explore the S. aureus population dynamics of each participant during the 28 day follow-up period. The isolate timelines are illustrated in Figure 3. Amongst the 17 par-ticipants, two common patterns were observed and reflected the way in which isolates had been selected. First, if a participant carried an azithromycin^R S. aureus at day 0 or day 3 (representing early/pre-intervention acquisition), the same clone was identified at day 28, suggesting that it had been maintained regardless of ST or the mac-rolide resistance gene identified during the 4 weeks post-intervention. This pattern was observed in all nine participants in groups 1 and 2 (Figure 3). Second, if a participant acquired an azithromycin^R S. aureus after day 3 (representing delayed acquisi-tion), then the first azithromycin^R clone acquired was an ST5 MSSA harbouring msr(A). This pattern was observed in seven of the eight participants in groups 3 and 4 (Figure 3). Further, this was also observed in the two mothers from groups 1 and 2 in which an azithromycin^S S. aureus was recovered at day 0 (pre-intervention) and an azithromycin^R S. aureus at day 3 (Figure 3, P593/6 and P592/ 5). In two participants, it appeared that the azithromycin^R ST5 iso-late recovered at day 6 was replaced by either an azithromycin^S ST5 (P361/2) or an azithromycin^S ST15 (P263/1) isolate at day 14, which then switched back to the azithromycin^R ST5 isolate at day 28 (Figure 3). This could represent repeated clonal replacement in these participants, dual carriage of both clones (which would be missed by this study as only a single colony was sequenced from each sample) or, in the case of P361/2, in vitro plasmid loss. It is also unclear why the four participants in group 3 only carry azithromycin^S ST15 MSSA prior to acquiring azithromycin^R MSSA, whereas group 4 are more varied (Figure 3). Again, this is possibly due to the small sample size considered in this study or may reflect an unknown selection pres-sure promoting short-term carriage of the azithromycin^S ST15 clone in participants assigned to the azithromycin treatment arm.

Collectively these isolate timelines suggest that participants who received the azithromycin intervention either maintained the azithromycin^R MSSA, which they probably carried prior to the intervention, or acquired one, replacing an azithromycin^S ST15 MSSA, with the resulting azithromycin^R MSSA being of varied ST and carry-ing either msr(A) or erm(C). In participants who received the pla-cebo, only azithromycin^R ST5 MSSA was acquired, replacing various azithromycin^S MSSA populations. There are multiple potential explanations for this finding. The increased use of azithromycin may have significantly altered the local molecular epidemiology of S. aureus, favouring azithromycin^R clones. When combined with widespread transmission, this could explain the high prevalence of the ST5 lineage if it additionally has a colonization advantage over other azithromycin^R clones. The absence of this lineage in the babies sampled at 1 year post-intervention suggests that any col-onization advantage, if indeed present, is only advantageous in the presence of high azithromycin use. Without a snapshot of the mo-lecular epidemiology of S. aureus in the catchment area prior to the trial, limited conclusions can be drawn about the impact that the trial has had on the S. aureus population and highlights the need for such information prior to any subsequent MDA campaigns.

Conclusions

We have used comprehensive genomic analyses to reveal the dynamics of azithromycin^R S. aureus colonization in mothers and babies after azithromycin treatment or placebo. Plasmid-encoded msr(A) in ST5 MSSA was the most common clone, being respon-sible for most azithromycin^R S. aureus acquisitions in both study arms. These results provide critical information to inform a greater understanding of the ecological impact of azithromycin prophy-laxis on staphylococcal populations in Western Africa.

Acknowledgements

We are grateful for assistance received from members of staff of the Microbiological Diagnostic Unit Public Health Laboratory, The University of Melbourne at The Peter Doherty Institute for Infection & Immunity. We thank the PregnAnZI team for the implementation of the trial, sam-ple collection and microbiological analysis. Also, the study participants are thanked for agreeing that further investigations could be carried out on their samples.

Funding

This work was jointly supported by the Medical Research Council (MRC) Unit The Gambia PhD fellowship and Doherty Applied Microbial Genomics, Department of Microbiology & Immunology, University of Melbourne at The Peter Doherty Institute for Infection & Immunity. The main trial was jointly funded by the UK MRC and the UK Department for International Development (DFID) under the MRC/DFID Concordat agreement (reference number MR/J010391/1) and is also part of the EDCTP2 programme (where EDCTP stands for European & Developing Countries Clinical Trials Partnership) supported by the European Union. B. P. H. (GNT1105905) and T. P. S. (GNT1008549) are supported by the National Health and Medical Research Council of Australia.

Transparency declarations

None to declare.

Author contributions

The project was conceived by A. R., B. P. H. and A. B. A. B. performed the bulk of the experimental work, with additional work performed by S. L. B., L. D., R. G., K. S., C. H. and O. S. A. B. and S. L. B. performed genomic analyses, with additional input from M. B. S., A. G. d. S. and T. P. S. C. B. assisted with statistical analyses. T. S. provided genomic analysis tools. A. B., S. L. B. and B. P. H. drafted the manuscript, with input from all co-authors. All authors approved the final version of the manuscript.

Supplementary data

Supplementary Materials and methods, Table S1, Figure S1 and Datasets S1 to S3 are available as Supplementary data at JAC Online.

References

1 Parnham MJ, Erakovic Haber V, Giamarellos-Bourboulis EJ et al. Azithromycin: mechanisms of action and their relevance for clinical applica-tions. Pharmacol Ther 2014; 143: 225–45.

2 Bignell C, Garley J. Azithromycin in the treatment of infection with Neisseria gonorrhoeae. Sex Transm Infect 2010; 86: 422–6.

3 Harding-Esch EM, Sillah A, Edwards T et al. Mass treatment with azithro-mycin for trachoma: when is one round enough? Results from the PRET trial in The Gambia. PLoS Negl Trop Dis 2013; 7: e2115.

4 Yohannan J, Munoz B, Mkocha H et al. Can we stop mass drug administra-tion prior to 3 annual rounds in communities with low prevalence of trach-oma? PRET Ziada trial results. JAMA Ophthalmol 2013; 131: 431–6.

5 Gebre T, Ayele B, Zerihun M et al. Comparison of annual versus twice-yearly mass azithromycin treatment for hyperendemic trachoma in Ethiopia: a cluster-randomised trial. Lancet 2012; 379: 143–51.

6 Schemann JF, Guinot C, Traore L et al. Longitudinal evaluation of three azithromycin distribution strategies for treatment of trachoma in a sub-Saharan African country, Mali. Acta Tropica 2007; 101: 40–53.

7 Lee JS, Munoz BE, Mkocha H et al. The effect of multiple rounds of mass drug administration on the association between ocular Chlamydia trachoma-tis infection and follicular trachoma in preschool-aged children. PLoS Negl Trop Dis 2014; 8: e2761.

8 Biebesheimer JB, House J, Hong KC et al. Complete local elimination of in-fectious trachoma from severely affected communities after six biannual mass azithromycin distributions. Ophthalmology 2009; 116: 2047–50.

9 Burr SE, Milne S, Jafali J et al. Mass administration of azithromycin and Streptococcus pneumoniae carriage: cross-sectional surveys in the Gambia. Bull World Health Organ 2014; 92: 490–8.

10 Porco TC, Gebre T, Ayele B et al. Effect of mass distribution of azithromycin for trachoma control on overall mortality in Ethiopian children: a randomized trial. JAMA 2009; 302: 962–8.

11 Keenan JD, Bailey RL, West SK et al. Azithromycin to reduce childhood mortality in sub-Saharan Africa. N Engl J Med 2018; 378: 1583–92.

12 Roca A, Oluwalana C, Bojang A et al. Oral azithromycin given during la-bour decreases bacterial carriage in the mothers and their offspring: a double-blind randomized trial. Clin Microbiol Infect 2016; 22: 565.e1–9.

13 Unger HW, Aho C, Ome-Kaius M et al. Impact of intermittent preventive treatment in pregnancy with azithromycin-containing regimens on maternal nasopharyngeal carriage and antibiotic sensitivity of Streptococcus pneumo-niae, Haemophilus influenzae, and Staphylococcus aureus: a cross-sectional survey at delivery. J Clin Microbiol 2015; 53: 1317–23.

14 Fessler AT, Wang Y, Wu C et al. Mobile macrolide resistance genes in staphylococci. Plasmid 2018; 99: 2–10.

15 Westh H, Hougaard DM, Vuust J et al. Prevalence of erm gene classes in erythromycin-resistant Staphylococcus aureus strains isolated between 1959 and 1988. Antimicrob Agents Chemother 1995; 39: 369–73.

16 Schmitz FJ, Sadurski R, Kray A et al. Prevalence of macrolide-resistance genes in Staphylococcus aureus and Enterococcus faecium isolates from 24 European university hospitals. Antimicrob Agents Chemother 2000; 45: 891–4.

17 Ross JI, Eady EA, Cove JH et al. Inducible erythromycin resistance in staphylococci is encoded by a member of the ATP-binding transport super-gene family. Mol Microbiol 1990; 4: 1207–14.

18 Leclercq R. Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implications. Clin Infect Dis 2002; 34: 482–92.

19 Matsuoka M, Inoue M, Endo Y et al. Characteristic expression of three genes, msr(A), mph(C) and erm(Y), that confer resistance to macrolide antibiotics on Staphylococcus aureus. FEMS Microbiol Lett 2003; 220: 287–93.

20 Prunier AL, Malbruny B, Tande D et al. Clinical isolates of Staphylococcus aureus with ribosomal mutations conferring resistance to macrolides. Antimicrob Agents Chemother 2002; 46: 3054–6.

21 Bojang E, Jafali J, Perreten V et al. Short-term increase in prevalence of nasopharyngeal carriage of macrolide-resistant Staphylococcus aureus fol-lowing mass drug administration with azithromycin for trachoma control. BMC Microbiol 2017; 17: 75.

22 Schaumburg F, Alabi AS, Peters G et al. New epidemiology of Staphylococcus aureus infection in Africa. Clin Microbiol Infect 2014; 20: 589–96.

23 Senghore M, Bayliss SC, Kwambana-Adams BA et al. Transmission of Staphylococcus aureus from humans to green monkeys in The Gambia as revealed by whole-genome sequencing. Appl Environ Microbiol 2016; 82: 5910–7.

24 Ebruke C, Dione MM, Walter B et al. High genetic diversity of Staphylococcus aureus strains colonising the nasopharynx of Gambian villag-ers before widespread use of pneumococcal conjugate vaccines. BMC Microbiol 2016; 16: 38.

25 Bojang A, Camara B, Jagne Cox I et al. Long-term impact of oral azithro-mycin taken by Gambian women during labour on prevalence and antibiotic susceptibility of S. pneumoniae and S. aureus in their infants: follow up of a randomized clinical trial. Clin Infect Dis 2018; 67: 1191–7.

26 Roca A, Oluwalana C, Camara B et al. Prevention of bacterial infections in the newborn by pre-delivery administration of azithromycin: study protocol of a randomized efficacy trial. BMC Pregnancy Childbirth 2015; 15: 302.

27 Drinkovic D, Fuller ER, Shore KP et al. Clindamycin treatment of Staphylococcus aureus expressing inducible clindamycin resistance. J Antimicrob Chemother 2001; 48: 315–6.

28 Hisatsune J, Hagiya H, Shiota S et al. Complete genome sequence of sys-temically disseminated sequence type 8 staphylococcal cassette chromosome mec type IVl community-acquired methicillin-resistant Staphylococcus aureus. Genome Announc 2017; 5: e00852-17.

29 Sugai M, Enomoto T, Hashimoto K et al. A novel epidermal cell differenti-ation inhibitor (EDIN): purification and characterization from Staphylococcus aureus. Biochem Biophys Res Commun 1990; 173: 92–8.

30 Argemi X, Nanoukon C, Affolabi D et al. Comparative genomics and identification of an enterotoxin-bearing pathogenicity island, SEPI-1/SECI-1, in Staphylococcus epidermidis pathogenic strains. Toxins 2018; 10: E93.

31 Helle L, Kull M, Mayer S et al. Vectors for improved Tet repressor-dependent gradual gene induction or silencing in Staphylococcus aureus. Microbiology 2011; 157: 3314–23.

Supplementary data

Supplementary Materials and Methods

PregnAnZI trial

The PregnAnZI trial recruited women delivering at the Bundung Maternal and Child Health Hospital (BMCHH) formerly called Jammeh Foundation for Peace (JFP), a government-run health facility located in Western Gambia that manages on average 4,500 deliveries per year. The population of the catchment area of (BMCHH) includes the main ethnic groups in The Gambia and the illiteracy rate is high. Details of the study protocol have been described elsewhere [1]. In brief, it was a phase-III, double-blind, placebo-controlled trial where 829 pregnant women attending the labour ward in the study health facility were randomized to receive a single oral dose of 2g of either oral AZI or a placebo (ratio 1:1). Recruitment of trial participants started in April 2013 and lasted 12 months, with an additional 2 months of follow-up. Study participants were monitored during eight weeks following the intervention and nasopharyngeal swabs (NPS) where collected during the first 4 weeks of the follow-up (day 0 for mothers, and day 3, 6, 14, and 28 for mothers and babies). Other biological samples were also collected during the follow-up period but were not considered for this genomic study [2]. All study mothers had signed consent during their pre-natal visits before being enrolled into the trial. The trial was approved by the Joint MRC/Gambia Government Ethics Committee.

Sample selection

In order to explore the potential genetic diversity amongst AZI^R *S. aureus* recovered during the trial, we stratified isolates collected at day 3 and day 28 into four groups (Figure 1). Two groups represented samples from participants assigned to the AZI treatment arm where an AZI^R *S. aureus* was recovered at both time points (Group 1) or only at day 28, with an AZI susceptible (AZI^S) *S. aureus* identified at day 3 (Group 3). The other two groups represented the same microbiological division but for samples recovered from participants assigned to the placebo arm (AZI^R *S. aureus* at day 3 and day 28 [Group2], or AZI^S *S. aureus* at day 3 and AZI^R *S. aureus* at day 28 [Group 4]). A total of 17 participants (34 isolates) were selected from these four groups for this genomic study. To further explore the population dynamics of AZI^R *S. aureus* carriage over the follow-up period, all *S. aureus* recovered from the above subjects

at other time points (days 0, 6 and 14) were also included. Further detail about isolate selection is provided in Figure 1.

Whole Genome Sequencing

Genomic DNA was extracted using the GenElute™ Bacterial Genomic DNA Kit (Sigma). DNA libraries were prepared using the Nextera XT kit (Illumina) and sequenced on the NextSeq 500 (Illumina) using 2 × 150-bp chemistry. One isolate (S80062MN28) was subjected to long-read sequencing on the RS-II (Pacific Biosciences) using P6-C4 chemistry. All sequence data generated for this study has been made publicly available through the European Nucleotide Archive, project accession PRJEB31151.

Bioinformatic Analysis

Short-read sequence data was assembled using SPAdes v3.11.1 [3], and contigs having a coverage less than 5 were excluded as contaminants. Isolate S80062MN28 was assembled from both long and short read sequence with Unicycler v0.4.6 in "bold" mode [4]. Annotation of plasmid pS80062MN28 was performed using Prokka v1.13.3[5], using plasmid pJSA01 (accession AP014922.1) [6]. Comparative alignment of plasmid sequences was performed using the Artemis Comparison Tool v17.0.1, excluding matches ≤ 100 bp in length[7]. *In silico* MLST and antibiotic resistance gene detection was performed on draft assemblies and complete genomes using mlst v2.10 (https://github.com/tseemann/mlst) and abricate v0.8.10 (https://github.com/tseemann/abricate), using the NCBI antimicrobial resistance database (retrieved 20-Sept-2018) and the virulence factor database (retrieved 14-Aug-2018).

For the phylogenetic analysis, short-read sequence data was aligned to the complete genomes of six *S. aureus* reference genomes (genome accessions: CP026964.1 [ST1]; CP026968.1 [ST5]; CP012970.1 [ST8]; LS483319.1 [ST15]; CP024998.1 [ST152]; LS483314.1 [ST669]), each selected for being the most closely related complete genome for each ST identified in the study population. Variant calling was achieved using Snippy v4.3.5 (https://github.com/tseemann/snippy). Whole genome alignments were used to construct maximum likelihood (ML) phylogenetic trees with IQtree v1.6.5 [8]. Model selection was used to find the best nucleotide substitution model for each alignment (chosen according to the Bayesian Information Criterion): this was either the HKY+F for ST5 and ST15 [9], or the F81+F

for ST1, ST8, ST152 and ST669 [10]. Alignments composed of greater than 5 isolates (ST5 and ST15) were subjected to 1000 ultra-fast bootstrap replicates [11]. ML trees were visualized using FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). Core pairwise SNP distances were calculated using an in-house pearl script.

The global *S. aureus* phylogeny was constructed using publicly available genomes and metadata as described in Guerillot *et al* [12]. The 66 isolates sequenced as part of this study were added, together with 28 newly sequenced *S. aureus* genomes from Africa (downloaded from the Staphopia database[13]. The 7191 genomes were mapped to the fully assembled reference genome *S. aureus* NRS384 and core genome alignment of the global collection (n=7192), all CC5 isolates (n=2735) or all CC15 isolates (n=179) were generated with snippy v4.3.6. Maximum likelihood phylogenetic trees were inferred with FastTree v2.1.8 using the generalized time-reversible (GTR) model. Trees were plotted and annotated using the R library *ggtree*[14]. Sub-trees of CC5 and CC15 were generated from the full CC5 or CC15 phylogenetic trees by extracting only isolates with a tip distance of < 23 nodes from the isolates sequenced in this study.

Detection of mutations in ribosomal proteins (*rplD*, *rplV*, and 23S rRNA) were performed using Snippy v4.3.8. Because of the multi-copy nature of the 23S rRNA, mutations were assessed by mapping short-read sequence data to a single copy of the gene from CP026968.1 [ST5] and calling mutations at a minimum mapped-base frequency of 0.5 (option "--min-frac 0.5"). Mutations were not called at a lower frequency as it was expected that they would need to be present in the majority of 23S rRNA copies to impact of phenotypic resistance[15, 16].

Cloning and transformation of *erm*C and *msr(A)* genes

Vector pRAB11 was initially amplified using primers pRAB11_F and pRAB11_R to create a backbone for SLiCE (seamless ligation cloning extract) cloning (all primer sequences are provided in Supplementary Dataset 2)[17]. The *erm*C and *msr(A)* genes were then amplified using primers *erm*C_pRAB11_F and *erm*C_pRAB11_R, and *msr(A)*_Saur_pRAB_F and *msr(A)*_Saur_pRAB_R, respectively. The PCR fragments were ligated using SLiCE and transformed into IM08B competent*E. coli* cells[18] . The transformed cells were plated on Luria broth (LB) agar with 100µg/ml ampicillin and incubated overnight at 37ºC. Successful

transformation was confirmed by colony PCR. The confirmed colonies were cultured overnight in LB with 100µg/ml ampicillin and plasmids extracted using QIAGEN mini prep kit. The empty vector or those containing *erm*C or *msr(A)* were electroporated into an ST5 A ZI^S *S. aureus* strain (S70065MN00) and plated on LB agar with 10µg/ml chloramphenicol before incubating overnight. Colonies were screen to confirm the presence of either *ermC* or *msr(A)* genes using the above primers. Antimicrobial susceptibility was tested as outlined below.

Bacterial conjugation

The donor (S80062MN28; azithromycin MIC 192 µg/ml, rifampicin MIC <1 µg/ml; fusidic acid MIC <1 µg/ml) and recipient strain that was induced to high level rifampicin and fusidic acid resistance through serial passage (S70065MN00, azithromycin MIC 1 µg/ml, rifampicin MIC >32 µg/ml; fusidic acid MIC >32 µg/ml) were mixed on a membrane filter (Millipore) with a donor-to-recipient ratio of 1:1. Plates were incubated overnight at 37°C. The bacterial cells were resuspended in PBS and spread on BHI agar plates containing the appropriate antibiotics. To quantify conjugative transfer efficiencies, dilutions were plated on BHI media containing antibiotics for the selection of; donor cells, recipient cells and plasmid containing recipient cells. Transconjugants were selected on plates containing azithromycin, rifampicin and fusidic acid all at 32 µg/ml. All conjugation assays were performed in duplicate. Control conjugation experiments were performed with the donor strain only, and no spontaneous resistant colonies were observed. The resistance profile of donor, recipient and transconjugant are summarised in Table S1.

Antimicrobial susceptibility testing

Phenotypic susceptibility to AZI and erythromycin was determined using Etest (BioMérieux), performed as per the manufacturer's recommendations. Etest values < 4µg/ml were interpreted as sensitive , ≥ 8 µg/ml as resistant, and ≥ 4 µg/ml but < 8 µg/ml as intermediate, as per the CLSI guidelines [19].

Statistics

Fisher's exact test was used to compare the prevalence of sequence types or macrolide resistance genes between the azithromycin and placebo group. A p value of 0.05 was used

as the cutoff for statistical significance. All analyses were done using STATA/SE v12.1 (https://www.stata.com/).

References

1. Roca A, Oluwalana C, Camara B, Bojang A, Burr S, Davis TM, Bailey R, Kampmann B, Mueller J, Bottomley C *et al*: **Prevention of bacterial infections in the newborn by pre-delivery administration of azithromycin: Study protocol of a randomized efficacy trial**. *BMC pregnancy and childbirth* 2015, **15**:302.

2. Roca A, Oluwalana C, Bojang A, Camara B, Kampmann B, Bailey R, Demba A, Bottomley C, D'Alessandro U: **Oral azithromycin given during labour decreases bacterial carriage in the mothers and their offspring: a double-blind randomized trial**. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* 2016, **22**(6):565 e561-569.

3. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD *et al*: **SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing**. *Journal of computational biology : a journal of computational molecular cell biology* 2012, **19**(5):455-477.

4. Wick RR, Judd LM, Gorrie CL, Holt KE: **Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads**. *PLoS computational biology* 2017, **13**(6):e1005595.

5. Seemann T: **Prokka: rapid prokaryotic genome annotation**. *Bioinformatics* 2014, **30**(14):2068-2069.

6. Hisatsune J, Hagiya H, Shiota S, Sugai M: **Complete Genome Sequence of Systemically Disseminated Sequence Type 8 Staphylococcal Cassette Chromosome mec Type IVl Community-Acquired Methicillin-Resistant Staphylococcus aureus**. *Genome announcements* 2017, **5**(35).

7. Carver TJ, Rutherford KM, Berriman M, Rajandream MA, Barrell BG, Parkhill J: **ACT: the Artemis Comparison Tool**. *Bioinformatics* 2005, **21**(16):3422-3423.

8. Nguyen LT, Schmidt HA, von Haeseler A, Minh BQ: **IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies**. *Molecular biology and evolution* 2015, **32**(1):268-274.

9. Felsenstein J: **Evolutionary trees from DNA sequences: a maximum likelihood approach**. *Journal of molecular evolution* 1981, **17**(6):368-376.

10. Hasegawa M, Kishino H, Yano T: **Dating of the human-ape splitting by a molecular clock of mitochondrial DNA**. *Journal of molecular evolution* 1985, **22**(2):160-174.

11. Hoang DT, Chernomor O, von Haeseler A, Minh BQ, Vinh LS: **UFBoot2: Improving the Ultrafast Bootstrap Approximation**. *Molecular biology and evolution* 2018, **35**(2):518-522.

12. Guerillot R, Goncalves da Silva A, Monk I, Giulieri S, Tomita T, Alison E, Porter J, Pidot S, Gao W, Peleg AY *et al*: **Convergent Evolution Driven by Rifampin Exacerbates the Global Burden of Drug-Resistant Staphylococcus aureus**. *mSphere* 2018, **3**(1).

13. Petit RA, 3rd, Read TD: **Staphylococcus aureus viewed from the perspective of 40,000+ genomes**. *PeerJ* 2018, **6**:e5261.

14. Yu G, Lam TT, Zhu H, Guan Y: **Two Methods for Mapping and Visualizing Associated Data on Phylogeny Using Ggtree**. *Molecular biology and evolution* 2018, **35**(12):3041-3043.

15. Sharkey LK, Edwards TA, O'Neill AJ: **ABC-F Proteins Mediate Antibiotic Resistance through Ribosomal Protection**. *mBio* 2016, **7**(2):e01975.

16. Silver LL: **Challenges of antibacterial discovery**. *Clinical microbiology reviews* 2011, **24**(1):71-109.

17. Zhang Y, Werling U, Edelmann W: **Seamless Ligation Cloning Extract (SLiCE) cloning method**. *Methods in molecular biology* 2014, **1116**:235-244.

18. Monk IR, Tree JJ, Howden BP, Stinear TP, Foster TJ: **Complete Bypass of Restriction Systems for Major Staphylococcus aureus Lineages**. *mBio* 2015, **6**(3):e00308-00315.

19. Institute CaLS: **Performance Standards for Antimicrobial Susceptibility testing**. 2014, **M100**(S24).

Table S1. Resistance profile of strains used in conjugation experiments

Strain	**Azithromycin MIC** µg/ml	**Rifampicin MIC** µg/ml	**Fusidic acid MIC** µg/ml
Donor (S80062MN28)	192	<1	<1
Recipient[#]	1	>32	>32
Transconjugants	>32	>32	>32

[#]**Note.** Strain S70065MN00 induced to high level rifampicin and fusidic acid resistance through serial passage.

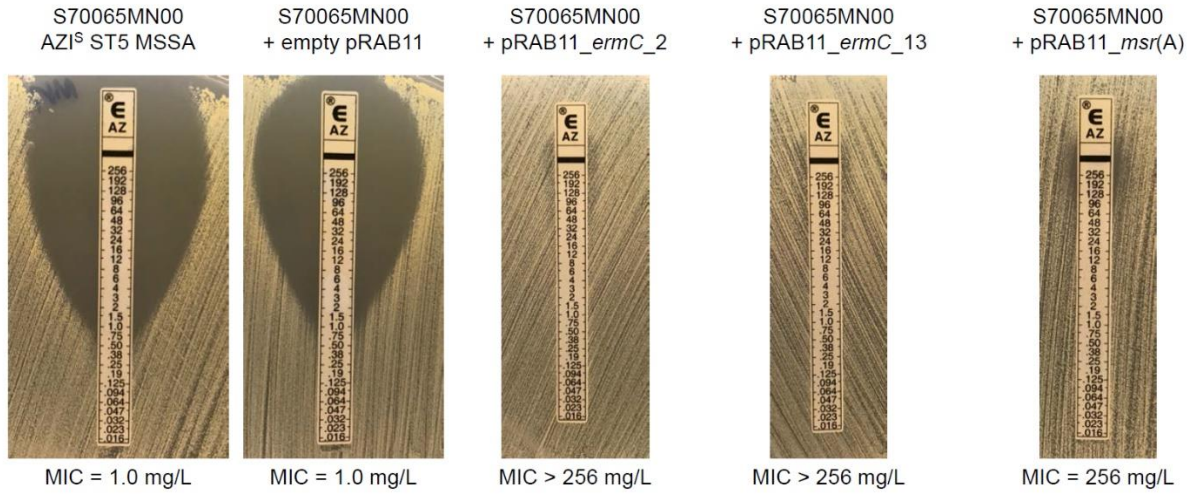


Figure S1. Phenotypic tests confirming that the acquisition of *ermC*_2, *ermC*_13 or *msr(A)* into a susceptible ST5 MSSA (representative of The Gambian lineage identified in this study) results in a significant increase in the minimum inhibitory concentration (MIC) to azithromycin.

CHAPTER 6

6.1 PhD Manuscript #3:

6.2 IMPACT OF INTRAPARTUM ORAL AZITHROMYCIN ON THE STAPHYLOCOCCAL ACQUIRED MACROLIDE RESISTOME OF INFANTS' NASOPHARYNX: A RANDOMISED CONTROLLED TRIAL

Published 23rd May 2020 in the *Clinical Infectious Disease Journal*

Impact of intrapartum oral azithromycin on the staphylococcal acquired macrolide resistome of infants' nasopharynx: a randomised controlled trial

Abdoulie Bojang[1#], Sarah L Baines[2], Bully Camara[1], Romain Guerillot[2], Liam Donovan[2], Raquel Sánchez Marqués[1], Ousman Secka[1], Umberto D'Alessandro[1], Christian Bottomley[3], Benjamin P Howden[2,4], Anna Roca[1#]

Affiliations

[1]Medical Research Council Unit The Gambia at the London School of Hygiene & Tropical Medicine, Fajara, The Gambia.

[2]Department of Microbiology & Immunology, The University of Melbourne at The Peter Doherty Institute for Infection & Immunity, Melbourne, Victoria, Australia.

[3]Tropical Epidemiology Group, London School of Hygiene & Tropical Medicine, United Kingdom

[4]Microbiological Diagnostic Unit Public Health Laboratory, Department of Microbiology & Immunology, The University of Melbourne at The Peter Doherty Institute for Infection & Immunity, Melbourne, Victoria, Australia.

Key words: Azithromycin, *erm*C, *msr(A)*, NPS, The Gambia

[#]Corresponding author:

Professor Anna Roca, Medical Research Council Unit The Gambia at the London School of Hygiene & Tropical Medicine, P. O. Box 273, Fajara, The Gambia. **Tel:** +2204495442 **ext.** 2305 **Fax:** +2204495919 Email: aroca@mrc.gm

[#]Alternate corresponding author:

Mr Abdoulie Bojang, Medical Research Council Unit The Gambia at the London School of Hygiene & Tropical Medicine, P. O. Box 273, Fajara, The Gambia. **Tel:** +2204495442 **ext.** 2118 **Fax:** +2204495919 Email: ambojang@mrc.gm

Running title: Nasopharyngeal Macrolide Resistome

Trial registration (main trial): ClinicalTrials.gov Identifier NCT01800942

Key points

Gambian children exposed to intrapartum azithromycin (2g) had higher prevalence and relative fluorescence of macrolide resistance genes (*msr(*A) and *erm*C) in the nasopharynx at the age of 28 days than non-exposed children but such difference disappeared by 12 months.

6.3 ABSTRACT

6.3.1 Background:

Several strategies using azithromycin prophylaxis proved effective in reducing maternal and neonatal morbidity/mortality. However, these interventions are associated with the emergence of azithromycin resistant bacterial species.

We conducted a phase III, double-blind, placebo-controlled randomized trial to determine the impact of one 2g oral dose of intrapartum azithromycin on bacterial colonization of Gambian women and their babies. In this *post hoc* analysis, we assess the impact of the intervention on macrolide resistance genes, specifically *msr(A)* and *ermC*, in the nasopharynx of study infants.

6.3.2 Methods:

Nasopharyngeal swabs (NPS) were collected at birth, 28 days and 12 months. Genomic DNA was extracted from the NPS. PCRs were performed using primers designed to detect *msr(A)* and *erm*C. Products were analysed using QIAxcel advanced Screen gel 1.5.0.

6.3.3 Results:

The analysis included 936 NPS samples from 312 children. At birth, the prevalence of *msr(A)* was similar in both arms, increased in the azithromycin arm at day 28 (60.7% vs. 29.9% in the azithromycin and placebo arms, respectively; OR, 3.61 [95% CI, 2.20-5.93]), and again similar between arms at 12 months. Prevalence of *erm*C followed a similar pattern, with differences between arms only apparent at day 28 (63.9% vs. 45.9% in the azithromycin and placebo

arms, respectively; OR, 2.09 [95% CI, 1.29-3.37]). At day 28, presence of one resistance gene was associated with presence of the other resistance gene.

6.3.4 Conclusions:

Children exposed to azithromycin during labour have a short-term increased prevalence of macrolide resistance genes in the nasopharynx which could potentially lead to the spread of macrolide resistance.

6.4 INTRODUCTION

For decades, oral azithromycin has been used for trachoma elimination (1,2). More recently, prophylactic use of azithromycin has been investigated in low and middle income countries as an intervention to decrease infant mortality (3). Azithromycin given during pregnancy increases birth weight (4) and reduces preterm delivery (5); intrapartum azithromycin reduces maternal and neonatal infections (6), colonization of Gram-positive bacteria (7) and malnutrition at 12 months of age (8).

However, azithromycin interventions may increase the prevalence of macrolide-resistant bacterial species, even after the administration of a single dose (7). In The Gambia, three annual rounds of mass drug administration (MDA) with azithromycin were associated with a short and long-term (30 months) increase in both the prevalence of azithromycin-resistant and inducible macrolide lincosamides and streptogramin B (MLSB) resistant *S. aureus* (9). In Papua New Guinea, the proportion of azithromycin resistant *S. aureus* was five-fold higher among pregnant women who received azithromycin compared to a control group (10).

In our double-blinded placebo-controlled trial conducted in The Gambia (namely PregnAnZI trial), a single dose of oral azithromycin was administered during labour to assess the impact on bacterial colonization of Gram-positive bacterial species (*Staphylococcus aureus*, *Streptococcus pneumoniae* and group B *Streptococcus*) in both mothers and their offspring. The intervention significantly reduced bacterial colonization but increased the prevalence of azithromycin resistant *S. aureus* during the neonatal period. By day 28, prevalence of *S. aureus* azithromycin resistance was significantly higher in the intervention compared to placebo arm (16.7%vs 4.5%; $P < .001$) (7). The observed increase was probably the result of the high concentration of azithromycin in the breast-milk (11). The carriage of azithromycin resistant *S. aureus* strains waned by the age of 12 months (12).

Although, macrolide resistance is known to result from both gene acquisition or mutation including efflux genes (*mef*(A), *msr(D), msr*(A/B) and methylation genes (*ermA, ermB* and *ermC*), molecular analysis of the azithromycin resistant *S. aureus* strains isolated in the PregnAnZI trial from both mothers and babies in The Gambia revealed that the genetic determinants responsible for macrolide resistance were predominantly, if not exclusively, *msr(A)* and *erm*C in this population(13). Both *msr(A)* and *ermC* genes are carried by mobile genetic elements, hence horizontal spread between bacteria colonising the same ecological niche can take place through transformation, conjugation or transduction (14–16). A comprehensive genomic analysis was performed on *S. aureus* as the bacterium was found to have high rates of macrolide resistance among women given azithromycin and their children, and hence may play a greater role in transmission of macrolide resistance genes. In fact, *msr(A)* and *ermC* can also be found in other bacteria that colonises the nasopharynx such as *Staphylococcus*, *Bacillus subtilis*, *Streptococcus*, *Enterococcus*, *Corynebacterium and Pseudomonas species* (17–21). Subsequently, screening a single bacterium (i.e. *S. aureus*) isolated from a sample to determine prevalence of resistance following oral azithromycin interventions may underestimate the true prevalence. Macrolide resistance needs to be more accurately assessed by examining the prevalence of these two genes across all bacterial species in a large number of clinical samples. The aim of this *post hoc* study is to evaluate the effect of intrapartum azithromycin on the prevalence and relative fluorescence of the macrolide resistance genes (i.e. *msr(A)* and *ermC*) in the nasopharynx at different time points during infancy.

6.5 MATERIALS AND METHODS

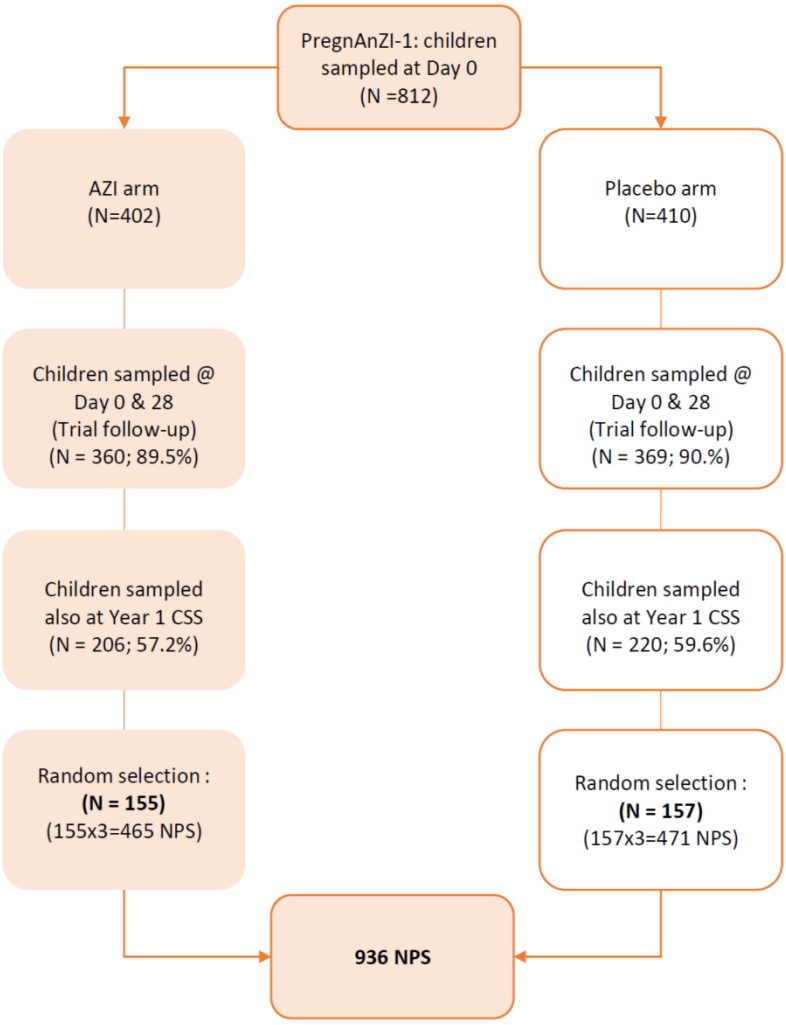
6.5.1 PregnAnZI trial and subsequent cross-sectional study (CSS)

The PregnAnZI trial was a phase-III, double-blind, placebo-controlled trial in which 829 pregnant women attending the labour ward were randomized to receive either a single oral dose (2g) of intrapartum azithromycin or placebo (ratio 1:1). The study protocol has been described elsewhere (22). Participants were recruited between April 2013 and April 2014. NPS were collected from babies at different time points during the neonatal period. In addition, NPS were collected in a follow up survey done when the children were between 11 and 13 months of age.

6.5.2 Sample selection for this post hoc study

Only children with NPS collected at birth (day 0), day 28 and at 12 months were eligible for inclusion in this *post hoc* study. A random sample of 312 children with the three samples (155 from the azithromycin and 157 from the placebo arm) were included in the current study, making a total of 936 samples (Figure **1**)**.**

Figure **1**. Flow chart showing how the analysis cohort was selected.



6.5.3 Sample collection and storage

NPS samples were collected by passing the tip of a calcium alginate swabs across the mucosa of the posterior wall of the nasopharynx and immediately inoculated into vials containing skim milk-tryptone-glucose-glycerol (STGG) transport medium (22). The vials were then sent to the laboratory for storage within 8 hours at -70C.

6.5.4 Ethical Approval

Both the main trial and the 12month survey were approved by the Joint Gambia Government/MRC Ethics Committee. Mothers of study children signed an informed consent for the trial and another one for the 12 months survey.

6.5.5 Laboratory method

6.5.5.1 DNA extraction

Genomic DNA was extracted directly from NPS using QIAamp DNA Mini Kit (QIAGEN, United Kingdom) protocol with some modifications. Frozen nasopharyngeal swabs in STGG medium were initially thawed at room temperature, vortexed briefly and 200µL of the sample added to 200µL of lysis buffer (TE buffer, lysozyme 30mg/ml, mutanolysin 50U/ml, lysostaphin 200µg/ml) before being incubated at 37°C for 1 hour. The manufacturer's protocol was followed for remainder of the extraction. The DNA was eluted in 100µl volume and stored at -20°C.

6.5.5.2 PCR

The primers for the amplification of the macrolide resistance genes [*msr(A)_F ATCCAATCATTGCACAAAATCTAACATT, msr(A)_R TAAATAGCTTCAAGTAAAGTTGTCTTACC* and *erm*C_F CTTGTTGATCACGATAATTTCCAAG, ermC_R TTGTATTCTTTGTTAACCCATTTCATAAC] were designed using Primer 3 and synthesized by Metabion, Germany. Primer sets were diluted to a final concentration of 10µM and assayed in 20µL PCR reaction volume containing 1µL of each primer, 10µL 2x Phire Green Hot Start II PCR (Thermo Scientific, United Kingdom) reaction buffer, 7µL nuclease water and 1µL of the genomic DNA. The cycling conditions included an initial denaturation at 98°C for 30 seconds, 35 cycles of denaturation at 98°C for 5 seconds, annealing at 52°C for 5 seconds and extension at 72°C for 10 seconds with a final extension at 72°C for 1 minute. Fully sequenced *S. aureus* carrying either the *erm*C or *msr(A)* genes were used as positive controls.

6.5.5.3 Electrophoresis

PCRs were performed alongside DNA extract from a pure *S. aureus* isolate known to carry either the *ermC* or *msr(A)* gene (positive controls) (13). The PCR products were analysed using QIAxcel advanced Screen gel 1.5.0 (QIAGEN, UK) (23) with a tolerance rate of ±15% of the expected band size (*msr(A)* 145bp and *erm*C 398bp). Results were categorized as negative or positive for macrolide resistance gene prevalence. In all the PCR reactions, the relative fluorescence unit (RFU) signal for the positive controls was greater than 1RFU as each *S. aureus* control was prepared from a pure laboratory culture and contained a high quantity of the PCR gene targets. Based on the above, amongst samples positive for *msr(A)* or *ermC*, an aleatory cut off was determined as low (≤1.00 RFU) or high (>1.00 RFU); used as a qualitative measurement to estimate the burden of macrolide-resistance bacteria in the sample.

6.5.5.4 Statistics

Pearson's χ2 test was used to compare the prevalence and relative fluorescence of macrolide resistance genes between arms at birth, day 28 and at 12 months. The χ2 test was also used to test for an association between the resistance genes at day 28. This analysis was stratified by trial arm, and a test for interaction was done to test whether the strength of association varied between arms. P-value ≤ 0.05 was used as the cut off for statistical significance. All analyses were carried out using Stata version 12.1 software.

6.6 RESULTS

6.6.1 Study population and samples

Baseline characteristics of the 312 study infants and their mothers selected for this *post hoc* study are shown in Table **1.**

Table **1.** Baseline characteristics of study mothers and babies

Characteristics	Azithromycin n= 155	Placebo n= 157	p value
Mother information			
Maternal age(years) at delivery, mean (SD)	26.7(5.4)[a]	26.9(5.0)	
Fundal height(cm), mean (SD)[b]	35.8(2.6)	35.8(3.3)	
Mode of delivery			
Vaginal	155(100)	157(100)	
Caesarean	0	0	
Ethnicity			0.405
Mandinka	68(43.9)	68(43.3)	
Jola	30(19.4)	26(16.6)	
Wollof	21(13.6)	14(8.9)	
Fula	18(11.6)	27(17.2)	
Sarahule	7(4.5)	5(3.2)	
Others	11(7.1)	17(10.8)	
Season of birth[c]			
Dry	114(73.6)	118(75.2)	0.745
Wet	41(26.5)	39(24.8)	
Child information at birth up to day 28			
Birthweight(kg), mean (SD)	3.13(0.5)	3.04(0.4)	
Apgar score[d]			
9 – 10	142(92.2)	147(93.6)	
7 - 8	12(7.8)	9(5.7)	
≤6	0	1(0.7)	
Twins	1(0.7)	4(2.6)	0.181
Singleton	154(99.3)	153(97.4)	
Sex[e]			
Female	83(53.9)	73(47.4)	0.254
Male	71(46.1)	81(52.6)	

Data are presented as No. (%) unless otherwise indicated.

Abbreviation: SD, standard deviation.

[a]one missing data item in the azithromycin arm.

[b]6 missing data (2 in the azithromycin arm and 4 in the placebo arm).

[c]wet season = June to October, dry season = November to May

[d]1 missing data in the azithromycin arm

[e]4 missing data (1 in azithromycin and 3 in placebo arm)

6.6.2 Prevalence of macrolide resistance (*msr(A)* and *erm*C) genes

Prevalence of *msr(A)* gene in infant NPS at birth was similar between trial arms (25.2% vs. 25.5% in the azithromycin and placebo arms, respectively). At day 28, prevalence of *msr(A)* gene was higher among children in the azithromycin arm (60.7% vs. 29.9%; OR, 3.61 [95% CI, 2.20-5.93]) but differences between arms waned by the age of 12 months (17.4% vs. 18.5% in the azithromycin and placebo arms, respectively; OR, 0.93 [95% CI, 0.50-1.73]). See Table **2** & Figure **2a**.

Prevalence of *erm*C gene in the infant NPS at birth was also similar between trial arms (30% vs. 35.0% in the azithromycin and placebo arms, respectively). At day 28, prevalence of *erm*C gene was higher among children in the azithromycin arm (63.9% vs. 45.9%; OR, 2.09 [95% CI, 1.29-3.37]) and again differences between arms waned by the age of 12 months (51.6% vs. 43.3% in the azithromycin and placebo arms, respectively; OR, 1.4 [95% CI, 0.87-2.24]). See Table **2** & Figure **2b.**

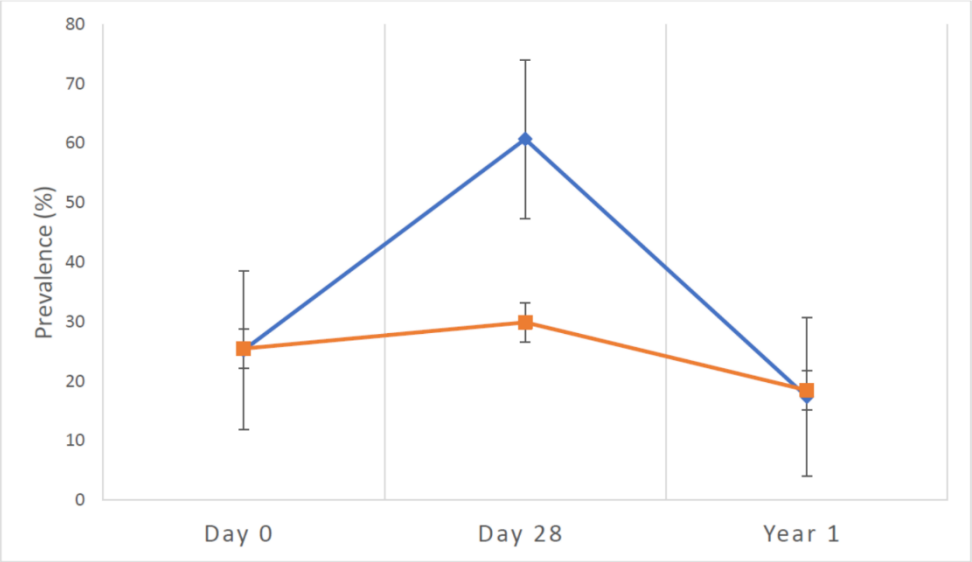
Table 2. Prevalence of msr(A) and ermC genes by Arm

	Day 0				Day 28				Year 1			
	AZI (%)	**P'bo (%)**	**OR (95% CI)**	**p value**	**AZI (%)**	**P'bo (%)**	**OR (95% CI)**	**p value**	**AZI (%)**	**P'bo (%)**	**OR (95% CI)**	**p value**
Gene	n = 155	n = 157			n = 155	n = 157			n = 155	n = 157		
msr(A)												
yes	39(25.2)	40(25.5)			94(60.7)	47(29.9)			27(17.4)	29(18.5)		
no	116(74.8)	117(74.5)	0.98(0.57-1.69)	0.949	61(39.3)	110(70.1)	3.61(2.20-5.93)	<0.001	128(82.6)	128(81.5)	0.93(0.50-1.73)	0.809
ermC												
yes	47(30.3)	55(35.0)			99(63.9)	72(45.9)			80(51.6)	68(43.3)		
no	108(69.7)	102(65.0)	0.81(0.49-1.33)	0.375	56(36.1)	85(54.1)	2.09(1.29-3.37)	0.001	75(48.4)	89(56.7)	1.40(0.87-2.24)	0.142

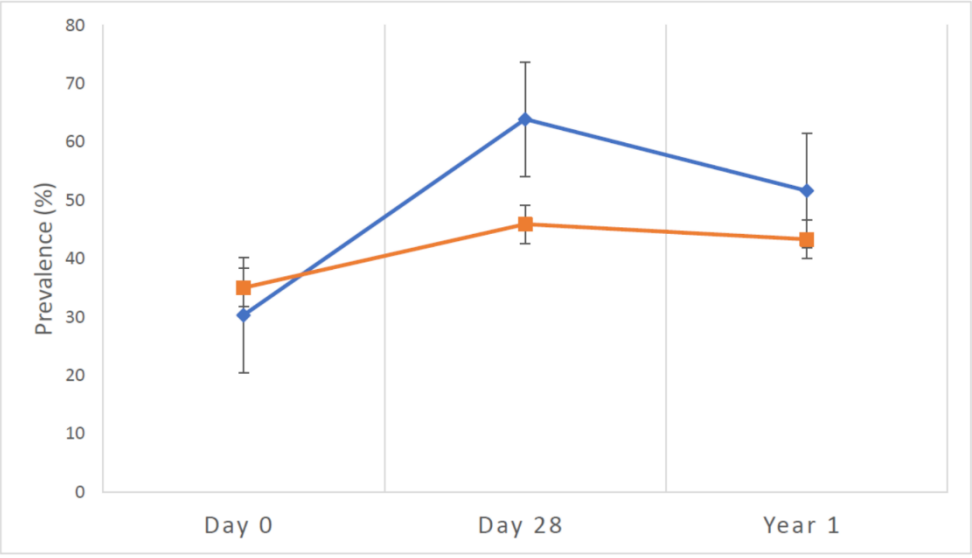
AZI = azithromycin P'bo = placebo CI = confidence interval

Figure **2.** Prevalence of macrolide resistance (**2a.**) *msr(A)* and (**2b.**) *erm*C genes in NPS over time and by trial arm. AZI arm (blue) and placebo arm (orange). Bars represent the 95%CI

2a.



2b.

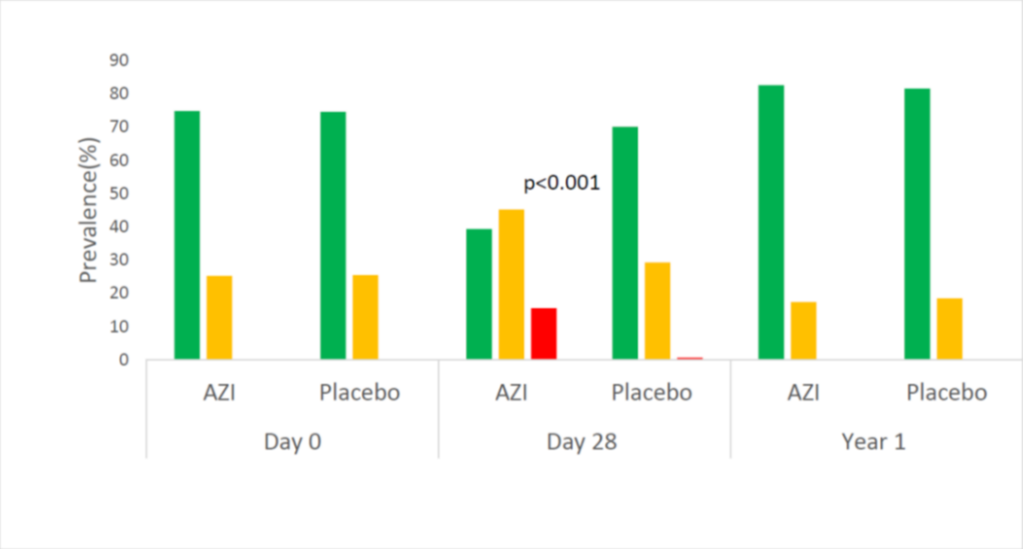


6.6.3 Relative fluorescence of macrolide resistance genes

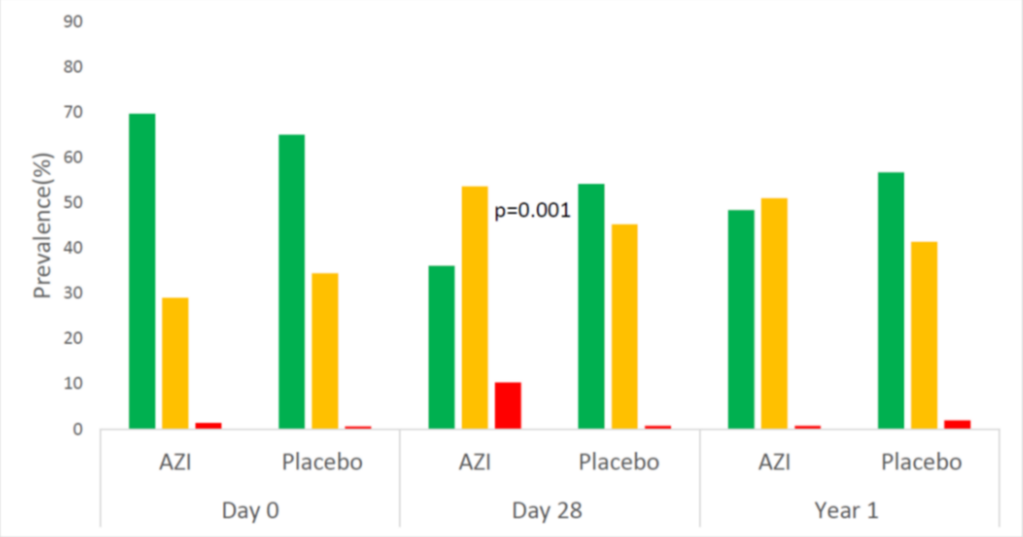
Samples with high relative fluorescence of *msr(A)* were only found at day 28. At that time point, the prevalence of high fluorescence signals was higher in samples from children in the azithromycin arm [15.5% vs. 0.6% among infants in the azithromycin and placebo arms respectively, OR 28.6 95% CI, 4.50-1182.43]) (Figure **3a**). Similarly, high relative fluorescence for *erm*C gene was observed at day 28 with higher prevalence among children in the azithromycin arm (10.3% vs. 0.6% in the azithromycin compared to placebo arm, OR 18.0 95% CI 2.70-757.60) (Figure **3b**).

Figure **3.** Relative fluorescence of macrolide resistance (**3a.**) *msr(A)* and (**3b.**) *erm*C genes in NPS over time and by arm. Samples negative for both resistant genes (green), low ≤ 1 relative fluorescence unit RFU (yellow) and high >1 RFU (red).

3a.



3b.



6.6.4 Association of *msr(A)* and *erm*C genes at day 28

At day 28, there was a positive association between *erm*C and *msr(A)* genes (OR 2.31 95%CI 1.42-3.76) Table **3**. Although the association appeared to be stronger in samples from the placebo arm, there was no evidence of an interaction by study arm (p=0.162).

Table 3. Association between msr(A) and ermC genes at Day 28 by arm

	ermC		OR	95% CI	p-value
Both arms	Pos n(%)	Neg n(%)			
msr(A)					
Pos	93(54.4)	48(34.0)			
Neg	78(45.6)	93(66.0)	2.31	1.42-3.76	<0.001
Azithromycin Arm					
msr(A)					
Pos	63(63.6)	31(55.4)			
Neg	36(36.6)	25(44.6)	1.41	0.68-2.90	0.311
Placebo Arm					
msr(A)					
Pos	30(41.7)	17(20.0)			
Neg	42(58.3)	68(80.0)	2.86	1.33-6.20	0.003

6.7 DISCUSSION

Our analysis shows that intrapartum azithromycin resulted in a short-term increased prevalence of both carriage and relative fluorescence of macrolide resistance genes (*msr(A)* and *ermC*) in the nasopharynx of study children. One year later, such difference had waned. The increased detection of the *msr(A)* and *erm*C genes at day 28 among children whose mothers received intrapartum azithromycin mirrors the reported phenotypic resistance for *S. aureus* at the same time point (7) that subsequently disappeared, also, by 12 months (12).

Our previous genomic analysis identified *msr(A)* as the dominant macrolide resistance gene in a sub-set of children carrying azithromycin-resistant *S. aureus* during the neonatal period, followed by *ermC* (13). Both *msr(A)* and *erm*C genes were present at all three time points in both arms. However, at day 28, the prevalence of macrolide resistance gene and relative fluorescence was highest among children whose mothers received intrapartum azithromycin for both *msr(A)* and *erm*C compared to children whose mothers received placebo. This finding suggested that exposure to azithromycin contributes to an increase in macrolide resistance gene carriage but also an increase in bacterial or resistance gene load.

Data on *msr(*A) gene prevalence following prophylactic azithromycin are scarce. In Australia and New Zealand, a non-significant increase in carriage of *msr(A)* was observed in patients with non-cystic fibrosis bronchiectasis on long-term (48 weeks) erythromycin (a sister macrolide to azithromycin) (24). However, in Niger children under 5 who received twice-a-year azithromycin had approximately, 30% higher prevalence of the streptococcal macrolide resistance determinant *mefA/E*, an equivalent of the staphylococcal *msr(*A) gene, than did children in the once-a-year group (25). Although formal comparisons between the aforementioned trial and our trial is difficult because of the different designs

(community seasonal prophylaxis versus single-dose intrapartum administration), it appears that *mef*A/E persisted longer within the population than *msr(A)* or *ermC*. We did not include *mef*A/E in our study as the gene is predominantly associated with *Streptococci*, and in our trial azithromycin resistance following intrapartum oral intervention was only significantly increased for *Staphylococcus* and not *Streptococcus* (7,12).

The increase in *erm*C prevalence observed at day 28 may be associated with increased macrolide resistance in the study population, including resistance to clindamycin and streptogramin B depending on whether the presence of the gene results in an inducible or constitutive phenotype (26,27). All *S. aureus* isolates carrying *erm*C from our previous molecular and phenotypic analysis showed constitutive resistance (12). Unlike *msr(A)* gene, the prevalence of *erm*C at 12 months was non-significantly higher in the azithromycin arm than in the placebo arm. Additional studies should determine whether the *erm*C gene, beyond being as prevalent as *msr(A)*, is also more persistent.

In our previous molecular analysis, we observed a negative association between the resistance genes whereby *S. aureus* isolates were positive for either *msr(A)* or *erm*C (13). The current analysis identified a positive association between the two genes. Taken together, these results indicate that in a given sample it is common for both macrolide resistance genes to be present but are likely carried by different bacterial isolates or species. For example *S. epidermidis* and *S. arlettea* are known to carry *ermC* and *msr(A)* respectively (20,21).

We also observed discrepancies between the current genotyping analysis and the former phenotyping in the prevalence of resistance. Most striking, the phenotypic prevalence of *S. aureus* resistance at day 0 was <1% compared to our 25%-35% for genotypic prevalence (7). It is important to note that these percentages are not comparable because phenotypic prevalence was estimated from cultured isolates of *S. aureus*, whereas genotypic

prevalence was estimated from detection of the macrolide resistance genes, irrespective of the bacterial species harbouring it. In fact, our findings suggest that most *msr(A)* and *ermC* genes found in the nose are carried by non *S. aureus* species and potential serve as a reservoir for macrolide resistance genes, underlying the importance of genotypic analysis of sample rather than individual bacteria to complement the more standard phenotypic methods.

Our study had some limitations. First, approximately half of the children that took part in the main trial were included in this study, although a significant selection bias is unlikely as most of the excluded children were beyond the upper age limit for inclusion. Second, only three time points were considered in this analysis, and therefore it is not possible to determine the length of time beyond 28 days in which the high prevalence of resistance in the azithromycin arm was maintained. Third, we did not use a housekeeping gene or control for the quality of DNA during the extraction. We do not consider that this has greatly affected the overall result as consistent methodology was applied to all samples in terms of collection, extraction of DNA and molecular testing. Fourth, the DNA concentration was not normalised prior to molecular testing. Therefore, we have interpreted the RFU values as suggestive of a difference in bacterial or gene load in the samples. Further, we only compared the relative fluorescence of the same gene between samples as the primers may have different amplification efficiency, as previously done by Keenan J D. *et al* (25). In addition, the clinical significance of detecting a macrolide resistance gene from an NPS for clinical care, in the absence of knowing whether the gene is carried by a pathogenic or commensal species, is not known. Finally, it was not possible to ascertain from the PCR used to detect *msr(A)* and *ermC* whether the genes were functional.

6.8 CONCLUSION

Children whose mothers were exposed to azithromycin during labour had increased prevalence and higher relative fluorescence of macrolide resistance genes (*msr(A)* and *erm*C) during the neonatal period but not at 12 months of age. Such results indicate a potential risk of antibiotic resistance genes spreading within the population during the neonatal period and highlights the importance of macrolide resistance genes surveillance in the event that prophylactic azithromycin interventions are rolled out.

6.9 ACKNOWLEDGEMENT

The authors are grateful to the field team for assisting with sample collection and to the study participants for agreeing to take part in the study. Special thanks to Kaddy Saho for helping with the laboratory analysis and Abdoulie Jabang for technical support in the use of the QIAxcel and Alyson L. Lush for helping with the design of Figure 1.

6.10 FUNDING

This work was jointly supported by the Medical Research Council Unit The Gambia at the London School of Hygiene and Tropical Medicine PhD fellowship. The main trial was jointly funded by the UK MRC and the UK Department for International Development (DFID) under the MRC/DFID Concordat agreement (reference number MR/J010391/1) and is also part of the EDCTP2 program supported by the European Union.

6.11 CONFLICT OF INTEREST

Authors have no conflicts of interest to declare. The project was conceived by AR, BPH and AB. BC collected samples from the main trial. AB performed the bulk of the experimental work, with additional work performed by SLB, RSM and OS. AB, LD and SLB designed the primers, with additional input from RG. CB assisted with statistical analyses. AB and AR drafted the manuscript, with input from all co-authors. All authors approved the final version of the manuscript.

6.12 REFERENCES

1. Harding-Esch EM, Sillah A, Edwards T, Burr SE, Hart JD, Joof H, et al. Mass Treatment with Azithromycin for Trachoma: When Is One Round Enough? Results from the PRET Trial in The Gambia. PLoS Negl Trop Dis. 2013;7(6):1–12.

2. Last AR, Burr SE, Harding-Esch E, Cassama E, Nabicassa M, Roberts CH, et al. The impact of a single round of community mass treatment with azithromycin on disease severity and ocular Chlamydia trachomatis load in treatment-naïve trachoma-endemic island communities in West Africa. Parasites and Vectors. 2017;10(1):1–11.

3. Keenan JD, Bailey RL, West SK, Arzika AM, Hart J, Weaver J, et al. Azithromycin to Reduce Childhood Mortality in Sub-Saharan Africa. N Engl J Med. 2018 Apr 25;378(17):1583–92.

4. Unger HW, Wangnapi RA, Ome-Kaius M, Boeuf P, Karl S, Mueller I, et al. Azithromycin-containing intermittent preventive treatment in pregnancy affects gestational weight gain, an important predictor of birthweight in Papua New Guinea – an exploratory analysis. Matern Child Nutr. 2016;12(4):699–712.

5. Luntamo M, Kulmala T, Mbewe B, Cheung YB, Maleta K, Ashorn P. Effect of repeated treatment of pregnant women with sulfadoxine- pyrimethamine and azithromycin on preterm delivery in Malawi: A randomized controlled trial. Am J Trop Med Hyg. 2010;83(6):1212–20.

6. Oluwalana C, Camara B, Bottomley C, Goodier S, Bojang A, Kampmann B, et al. Azithromycin in Labor Lowers Clinical Infections in Mothers and Newborns: A Double-Blind Trial. Pediatrics. 2017 Feb;139(2):e20162281.

7. Roca A, Oluwalana C, Bojang A, Camara B, Kampmann B, Bailey R, et al. Oral azithromycin given during labour decreases bacterial carriage in the mothers and their offspring: a double-blind randomized trial. Clin Microbiol Infect. 2016 Jun 1;22(6):565.e1-565.e9.

8. Roca A, Camara B, Oluwalana C, Lette K, Bottomley C, D'Alessandro U. Long-lasting effect of oral azithromycin taken by women during labour on infant nutrition: Follow-up cohort of a randomized clinical trial in western Gambia. PLoS One. 2018;13(10):1–9.

9. Bojang E, Jafali J, Perreten V, Hart J, Harding-Esch EM, Sillah A, et al. Short-term increase in prevalence of nasopharyngeal carriage of macrolide-resistant Staphylococcus aureus following mass drug administration with azithromycin for trachoma control. BMC Microbiol. 2017;17(1):1–10.

10. Unger HW, Aho C, Ome-Kaius M, Wangnapi RA, Umbers AJ, Jack W, et al. Impact of intermittent preventive treatment in pregnancy with azithromycin-containing regimens on maternal nasopharyngeal carriage and antibiotic sensitivity of Streptococcus pneumoniae, Haemophilus influenzae, and Staphylococcus aureus: A cross-sectional. J Clin Microbiol. 2015;53(4):1317–23.

11. Salman S, Davis TME, Page-Sharp M, Camara B, Oluwalana C, Bojang A, et al. Pharmacokinetics of Transfer of Azithromycin into the Breast Milk of African Mothers. Antimicrob Agents Chemother. 2016 Mar 1;60(3):1592–9.

12. Bojang A, Camara B, Jagne Cox I, Oluwalana C, Lette K, Usuf E, et al. Long-term Impact of Oral Azithromycin Taken by Gambian Women during Labor on Prevalence and Antibiotic Susceptibility of Streptococcus pneumoniae and Staphylococcus aureus in Their Infants: Follow-up of a Randomized Clinical Trial.

Clin Infect Dis. 2018 Sep 28;67(8):1191–7.

13. Bojang A, Baines SL, Donovan L, Guerillot R, Stevens K, Higgs C, et al. Genomic investigation of Staphylococcus aureus recovered from Gambian women and newborns following an oral dose of intrapartum azithromycin. J Antimicrob Chemother. 2019;74(11):3170–8.

14. Brown-Jaque M, Calero-Cáceres W, Muniesa M. Transfer of antibiotic-resistance genes via phage-related mobile elements. Plasmid [Internet]. 2015;79:1–7. Available from: http://dx.doi.org/10.1016/j.plasmid.2015.01.001

15. Lerminiaux NA, Cameron ADS. Horizontal transfer of antibiotic resistance genes in clinical environments. Can J Microbiol. 2019;65(1):34–44.

16. Sørensen SJ, Bailey M, Hansen LH, Kroer N, Wuertz S. Studying plasmid horizontal transfer in situ: A critical review. Nat Rev Microbiol. 2005;3(9):700–10.

17. Ojo KK, Striplin MJ, Ulep CC, Close NS, Zittle J, Luis H, et al. Enterococcus , Corynebacterium , and Pseudomonas Isolates Antimicrob Agents Chemother.. 2006;50(3):1089–91.

18. Nawaz MS, Khan AA, Cerniglia CE. Detection of erythromycin resistant methylase gene by the polymerase chain reaction. Mol Cell Probes. 1997;11(5):317–22.

19. Jensen LB, Frimodt-Møller N, Aarestrup FM. Presence of erm gene classes in Gram-positive bacteria of animal and human origin in Denmark. FEMS Microbiol Lett. 1999;170(1):151–8.

20. Dziri R, Klibi N, Lozano C, Ben Said L, Bellaaj R, Tenorio C, et al. High prevalence of Staphylococcus haemolyticus and Staphylococcus saprophyticus in environmental samples of a Tunisian hospital. Diagn Microbiol Infect Dis [Internet].

2016;85(2):136–40. Available from: http://dx.doi.org/10.1016/j.diagmicrobio.2016.03.006

21. Juda M, Chudzik-Rzad B, Malm A. The prevalence of genotypes that determine resistance to macrolides, lincosamides, and streptogramins B compared with spiramycin susceptibility among erythromycin-resistant Staphylococcus epidermidis. Mem Inst Oswaldo Cruz. 2016;111(3):155–60.

22. Roca A, Oluwalana C, Camara B, Bojang A, Burr S, Davis TME, et al. Prevention of bacterial infections in the newborn by pre-delivery administration of azithromycin: Study protocol of a randomized efficacy trial. BMC Pregnancy Childbirth. 2015 Nov 19;15(1).

23. Förster I, Brockmann M, Schildgen O, Schildgen V. Microsatellite instability testing in colorectal cancer using the QiaXcel advanced platform. BMC Cancer. 2018;18(1):1–6.

24. Choo JM, Abell GCJ, Thomson R, Morgan L, Waterer G, Gordon DL, et al. Impact of Long-Term Erythromycin Therapy on the Oropharyngeal Microbiome and Resistance Gene Reservoir in Non-Cystic Fibrosis Bronchiectasis. mSphere. 2018 Apr 18;3(2):e00103-18.

25. Keenan JD, Chin SA, Amza A, Kadri B, Nassirou B, Cevallos V, et al. The Effect of Antibiotic Selection Pressure on the Nasopharyngeal Macrolide Resistome: A Cluster-randomized Trial. Clin Infect Dis. 2018 Nov 13;67(11):1743–9.

26. Weisblum B. Erythromycin resistance by ribosome modification. Antimicrob Agents Chemother. 1995;39(3):577–85.

27. Leclercq R. Mechanisms of Resistance to Macrolides and Lincosamides: Nature of

the Resistance Elements and Their Clinical Implications. Clin Infect Dis. 2002;34(4):482–92.

CHAPTER 7

7.1 GENERAL DISCUSSION

A proof-of-concept trial (namely PregnAnZI-1) showed that intrapartum oral azithromycin prophylaxis significantly reduced maternal and neonatal colonisation of Gram-positive bacteria and clinical infections [56, 140]. Consequently, the intervention is currently being evaluated in larger trials to assess efficacy in maternal and neonatal sepsis as well as neonatal mortality. However, efficacy results will need to be balanced with the effect of the intervention on antimicrobial resistance. Therefore, the future consideration of intrapartum oral azithromycin as a public health intervention will need a clear understanding of the impact on bacterial carriage and antimicrobial resistance.

In The Gambia, *S. aureus* is the main cause of bacteraemia in all age groups and the most prevalent cause of neonatal sepsis [11, 30, 31]. The bacterium has the potential to develop resistance to many antibiotics including macrolides following exposure. Intrapartum oral azithromycin prophylaxis has also been shown to result in increased prevalence of azithromycin resistance *S. aureus* shortly following exposure [56]. These investigations conducted as part of my PhD are important for future decisions on the use of oral azithromycin in labour to reduce maternal and neonatal mortality in the Gambia and beyond.

I have shown that although azithromycin resistant *S. aureus* increased significantly shortly following exposure among both mothers and their new-borns, the resistance waned by 12 months. At this time point, no differences were observed in the prevalence of carriage and antibiotic resistant bacteria between children whose mothers were exposed to azithromycin during labour compared to those children whose mothers had taken placebo [154]. Similarly, no differences were found between study arms in the prevalence of *S.*

aureus resistance to other antibiotics such as erythromycin, clindamycin, chloramphenicol, cefoxitin and cotrimoxazole. This is an important result as the above antibiotics are commonly used in The Gambia for the treatment of diseases cause by *S. aureus*.

The finding suggests that the intervention has no risk of azithromycin or other antibiotic resistant *S. aureus* strains persisting in the nasopharynx of study children following exposure. However, given an impact on resistance was observed in the short term, following the rollout of oral azithromycin prophylaxis to all pregnant women in labour, continuous monitoring will be required among exposed individuals but also their close contacts for the emergence of antibiotic resistance. This will be necessary as following the roll out, a large number of women and babies will be exposed to the antibiotic resulting in high antibiotic pressure within the communities. This may result in the emergence of macrolide resistance in bacterial species not observed during the trial. In the trial, the antibiotic pressure was low since only a small proportion of the pregnant women and their babies in the population were exposed to the antibiotic.

Understanding the genetic basis of *S. aureus* macrolide resistance is also very important as it permits a more detailed understanding of the impact of azithromycin exposure at the molecular level. It sheds light on the different macrolide resistance mechanisms used by bacteria. It also provides genetic information about the genes responsible for resistance and whether they are chromosomal or located on a mobile genetic element, which may have implications for the risk of dissemination of resistance in the population. Antibiotic resistance genes located on a mobile genetic element have the potential to transfer horizontally between bacteria of the same or different species colonising the same ecological niche. Hence, screening one colony grown from a sample to determine prevalence of macrolide resistance following oral azithromycin interventions may underestimate the real prevalence of resistance.

I have identified the two main genes (*msr(A)* and *ermC*) located on mobile genetic elements encoding protein products resulting in azithromycin resistance in our population. The *msr(A)* gene was the dominant gene associated with *S. aureus* macrolide resistance among all isolates recovered from mothers and babies in the study. All *S. aureus* isolates in the placebo arm carried the *msr(A)* gene suggesting the dominance of this gene type circulating in the population prior to the intervention. The importance of such finding is that it provides information about the mode of transmission and spread within the population as chromosomal genes transfer mainly from parent to daughter cells whereas genes located on mobile genetic elements are able to transfer horizontally between bacteria of same or different species through transformation, conjugation or transduction [155, 156]. Intrapartum oral azithromycin intervention appeared to select for the *ermC* gene as 36% of resistant *S. aureus* isolates from mothers and babies exposed to antibiotic carried the gene as opposed to 0% in the placebo arm. The selection of one gene type over the other does not appear to have any negative consequences as there was no evidence of induced macrolide resistance *S. aureus* associated with the *ermC* gene. The significance of the above findings is that I have been able to establish the genes predominantly associated with macrolide resistance among one of the most important bacterial species causing infections in The Gambian population and demonstrate how the prevalence of these genes was affected by the intervention. I have also been able to show that although the intervention appears to select one resistance gene type over the other, overall, it has no negative consequences as the resulting gene types were mainly constitutive indicating resistance to macrolide, lincosamides and streptogramin B antibiotics but susceptible to other antibiotics.

Further, I have shown the circulating *S. aureus* sequence types (STs) examined dominated by ST5 and the genetic relatedness of isolates from the same individuals at different time

points. ST5 was dominant among mothers and babies but the intervention appears to select for ST15 over ST5 among azithromycin exposed individuals. Although ST15 is more often associated with pathogenicity it is also more susceptible to antibiotic and lacks the backbone to carry the *mecA* gene that confers methicillin resistance [157]. *S. aureus* isolates coming from the same individual at different time points were genetically very closely related.

Previously, I established that both *msr(A)* and *ermC* genes were located on a mobile genetic element [158] with the potential of resistance genes transferring horizontally between bacteria. I then analysed prevalence in nasopharyngeal sample at three time points (at birth, day 28 and 12months) among infants from the azithromycin and placebo arms of the PregnAnZI-1 study. The PCR method used detected the presence of the genes regardless of the bacterial source.

I found that prevalence of the macrolide resistance genes (*msr(A)* and *ermC*) in the nasopharynx were significantly higher especially by day 28 among children exposed to intra partum azithromycin compared to those in the placebo group. The prevalence of azithromycin resistance genes was about 4 fold higher at day 28 in the nasopharynx of children exposed to azithromycin compared to the prevalence of *S. aureus* azithromycin resistance previously reported among the same subjects at the same time point [56]. It is important to note that these percentages are not comparable because phenotypic prevalence was estimated from cultured isolates of *S. aureus*, whereas genotypic prevalence was estimated from detection of the macrolide resistance genes, irrespective of the bacterial species harbouring it. In fact, our findings suggest that most *msr(A)* and *ermC* genes found in the nose are carried by non *S. aureus* species [124, 159-162] and potentially serve as a reservoir for macrolide resistance genes, underlying the importance of genotypic analysis of sample rather than individual bacteria to complement the more

standard phenotypic methods. In addition, molecular detection of antibiotic resistant genes directly from samples following azithromycin exposure is considered highly relevant for public health as an early "early warning" system compared with the slower expected changes in indicator pathogens from clinical samples [163]. Consistently, the relative fluorescence of both genes was also higher among children exposed to azithromycin compared to those in the placebo group with the peak at day 28 similar to what was observed for *S. aureus* resistance [56].

7.2 LIMITATIONS

A number of limitations apply to the above investigations. In assessing the long-term impact of the intervention on carriage and resistance, no samples were collected between day 28 of the main trial and the survey done at the infants' first birthday. Therefore, I was unable to determine how long *S. aureus* resistant isolates persisted in the population. Such information is important to determine the potential risk of resistance bacteria transmission and establishment within the population. In addition, I didn't assess if resistant strains are transmitted or not to close contacts beyond the mother and baby pair which is also important for establishing resistance at the community level.

For the genomic investigation of *S. aureus* strains from mothers and babies, the main limitation relates to the fact that only a single colony was picked from each nasopharyngeal sample. Individuals may be colonised by more than one *S. aureus* strain hence the potential "cloud of diversity" that may exist within the sample cannot be accounted for when using a single colony pick. This limits the interpretation of results in terms of understanding the diversity of *S. aureus* genotypes colonising mothers and babies at the different time points.

In the PCR detection of staphylococcal macrolide resistance genes, the DNA concentration was not normalised prior to molecular testing. Therefore, I interpreted the RFU values as

suggestive of a difference in bacterial or gene load in the samples rather than a quantitative measure of the different genes. The copy numbers of the different genes in the samples are bound to affect the signal intensity of the fluorescence. Again, this analysis used samples at day 0, 28 and 12 months and therefore there is a big gap between the latest two samples.

7.3 CONCLUSIONS

Emergence of antimicrobial resistant bacteria following intrapartum oral azithromycin exposure was of concern. All of the above findings indicate a short-term increase in the prevalence of azithromycin resistant *S. aureus* that waned 12 months following exposure. Further, there was no increase resistance to other commonly used antibiotics. In The Gambia, it is likely the prevalence of antimicrobial resistance will not persist following the rollout of intrapartum oral azithromycin prophylaxis to all pregnant women in labour as baseline azithromycin resistance in the country is very low. Although these findings are reassuring in a country like The Gambia where baseline azithromycin resistance was low, I don't know how they would translate to other countries with higher baseline resistance prevalence. If the intervention is implemented, continuous monitoring of antibiotic resistance will be necessary for *S. aureus* as well as other potentially pathogenic bacteria.

7.4 FUTURE DIRECTIONS

It has been established that prevalence of azithromycin resistance *S. aureus* increased up to day 28 but by 12months the resistance waned. Future studies should collect samples monthly from both mothers and babies between day 28 and 12 months post intervention in order to more accurately determine the exact time point when resistance disappeared.

The transmissibility of azithromycin resistant *S. aureus* strains that emerged shortly following azithromycin prophylaxis especially to close contact within the household should also be investigated

Azithromycin will probably not decrease carriage and may increase resistance to Gram-negative bacteria. Currently there are no data on the impact of intrapartum oral azithromycin on Gram-negative bacteria (e.g. *Escherichia coli*, *Pseudomonas aeruginosa, Klebsiella pneumoniae*). Future studies will need to investigate both the short and long-term impact of the intervention on the carriage and antibiotic resistance of these bacteria. Assessment of antibiotic resistance using phenotypic methods should always be completed by molecular testing of antibiotic resistance genes directly from the sample in order to obtain a more accurate measure of antibiotic resistance prevalence.

Following the successful implementation of the intrapartum oral azithromycin, continuous surveillance at population level should be conducted for colonisation by performing periodical cross-sectional studies of azithromycin resistance. Further, rates of azithromycin resistance bacteria from clinical samples collected from patients admitted at referral hospitals countrywide should be investigated.

Finally, a detailed investigation of the impact of the intervention on nasopharyngeal, skin, vaginal, break milk microbiomes of exposed and unexposed individuals using metagenomics approach in order to understand the effect on the microbial population.

7.6 REFERENCES

1. Aires de Sousa, M. and H. de Lencastre, *Bridges from hospitals to the laboratory: genetic portraits of methicillin-resistant Staphylococcus aureus clones.* FEMS Immunol Med Microbiol, 2004. **40**(2): p. 101-11.
2. Naber, C.K., *Staphylococcus aureus bacteremia: epidemiology, pathophysiology, and management strategies.* Clin Infect Dis, 2009. **48 Suppl 4**: p. S231-7.
3. Laupland, K.B., T. Ross, and D.B. Gregson, *Staphylococcus aureus bloodstream infections: risk factors, outcomes, and the influence of methicillin resistance in Calgary, Canada, 2000-2006.* J Infect Dis, 2008. **198**(3): p. 336-43.
4. Chu, V.H., et al., *Staphylococcus aureus bacteremia in patients with prosthetic devices: costs and outcomes.* Am J Med, 2005. **118**(12): p. 1416.
5. Keynan, Y. and E. Rubinstein, *Staphylococcus aureus bacteremia, risk factors, complications, and management.* Crit Care Clin, 2013. **29**(3): p. 547-62.
6. Boucher, H.W. and G.R. Corey, *Epidemiology of methicillin-resistant Staphylococcus aureus.* Clin Infect Dis, 2008. **46 Suppl 5**: p. S344-9.
7. Lowy, F.D., *Staphylococcus aureus infections.* N Engl J Med, 1998. **339**(8): p. 520-32.
8. Wisplinghoff, H., et al., *Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study.* Clin Infect Dis, 2004. **39**(3): p. 309-17.
9. Tong, S.Y., et al., *Staphylococcus aureus infections: epidemiology, pathophysiology, clinical manifestations, and management.* Clin Microbiol Rev, 2015. **28**(3): p. 603-61.
10. Nickerson, E.K., et al., *Staphylococcus aureus bacteraemia in a tropical setting: patient outcome and impact of antibiotic resistance.* PLoS One, 2009. **4**(1): p. e4308.
11. Odutola, A., et al., *Staphylococcus aureus Bacteremia in Children of Rural Areas of The Gambia, 2008-2015.* Emerg Infect Dis, 2019. **25**(4): p. 701-709.
12. Schaumburg, F., et al., *New epidemiology of Staphylococcus aureus infection in Africa.* Clin Microbiol Infect, 2014. **20**(7): p. 589-96.
13. Styers, D., et al., *Laboratory-based surveillance of current antimicrobial resistance patterns and trends among Staphylococcus aureus: 2005 status in the United States.* Ann Clin Microbiol Antimicrob, 2006. **5**: p. 2.
14. Klevens, R.M., et al., *Invasive methicillin-resistant Staphylococcus aureus infections in the United States.* JAMA, 2007. **298**(15): p. 1763-71.
15. Thomer, L., O. Schneewind, and D. Missiakas, *Pathogenesis of Staphylococcus aureus Bloodstream Infections.* Annu Rev Pathol, 2016. **11**: p. 343-64.
16. Mejer, N., et al., *Stable incidence and continued improvement in short term mortality of Staphylococcus aureus bacteraemia between 1995 and 2008.* BMC Infect Dis, 2012. **12**: p. 260.
17. Benfield, T., et al., *Increasing incidence but decreasing in-hospital mortality of adult Staphylococcus aureus bacteraemia between 1981 and 2000.* Clin Microbiol Infect, 2007. **13**(3): p. 257-63.
18. Larsen, M.V., et al., *Major but differential decline in the incidence of Staphylococcus aureus bacteraemia in HIV-infected individuals from 1995 to 2007: a nationwide cohort study*.* HIV Med, 2012. **13**(1): p. 45-53.
19. Asgeirsson, H., et al., *Staphylococcus aureus bacteraemia in Iceland, 1995-2008: changing incidence and mortality.* Clin Microbiol Infect, 2011. **17**(4): p. 513-8.
20. Lyytikainen, O., et al., *Trends and outcome of nosocomial and community-acquired bloodstream infections due to Staphylococcus aureus in Finland, 1995-2001.* Eur J Clin Microbiol Infect Dis, 2005. **24**(6): p. 399-404.
21. Jacobsson, G., et al., *The epidemiology of and risk factors for invasive Staphylococcus aureus infections in western Sweden.* Scand J Infect Dis, 2007. **39**(1): p. 6-13.
22. van Cleef, B.A., et al., *Cross border comparison of MRSA bacteraemia between The Netherlands and North Rhine-Westphalia (Germany): a cross-sectional study.* PLoS One, 2012. **7**(8): p. e42787.

23. Rovira, G., et al., *The Challenge of Diagnosing and Treating Staphylococcus aureus Invasive Infections in a Resource-limited Sub-Saharan Africa Setting: A Case Report.* J Trop Pediatr, 2015. **61**(5): p. 397-402.
24. Darboe, S., et al., *Community-acquired Invasive Bacterial Disease in Urban Gambia, 2005-2015: A Hospital-based Surveillance.* Clin Infect Dis, 2019. **69**(Supplement_2): p. S105-S113.
25. Seydi, M., et al., *[Staphylococcus aureus bacteremia in the Dakar Fann University Hospital].* Med Mal Infect, 2004. **34**(5): p. 210-5.
26. Sigauque, B., et al., *Community-acquired bacteremia among children admitted to a rural hospital in Mozambique.* Pediatr Infect Dis J, 2009. **28**(2): p. 108-13.
27. Berkley, J.A., et al., *Bacteremia among children admitted to a rural hospital in Kenya.* N Engl J Med, 2005. **352**(1): p. 39-47.
28. Nielsen, M.V., et al., *Incidence and characteristics of bacteremia among children in rural Ghana.* PLoS One, 2012. **7**(9): p. e44063.
29. Okomo, U., et al., *Aetiology of invasive bacterial infection and antimicrobial resistance in neonates in sub-Saharan Africa: a systematic review and meta-analysis in line with the STROBE-NI reporting guidelines.* Lancet Infect Dis, 2019. **19**(11): p. 1219-1234.
30. Hill, P.C., et al., *Bacteraemia in patients admitted to an urban hospital in West Africa.* BMC Infect Dis, 2007. **7**: p. 2.
31. Secka, F., et al., *Bacteremia in Childhood Life-Threatening Infections in Urban Gambia: EUCLIDS in West Africa.* Open Forum Infect Dis, 2019. **6**(9): p. ofz332.
32. Peacock, S.J., et al., *Determinants of acquisition and carriage of Staphylococcus aureus in infancy.* J Clin Microbiol, 2003. **41**(12): p. 5718-25.
33. Andersen, P.S., et al., *Risk factors for Staphylococcus aureus nasal colonization in Danish middle-aged and elderly twins.* Eur J Clin Microbiol Infect Dis, 2013. **32**(10): p. 1321-6.
34. Sakr, A., et al., *Staphylococcus aureus Nasal Colonization: An Update on Mechanisms, Epidemiology, Risk Factors, and Subsequent Infections.* Front Microbiol, 2018. **9**: p. 2419.
35. Sollid, J.U., et al., *Staphylococcus aureus: determinants of human carriage.* Infect Genet Evol, 2014. **21**: p. 531-41.
36. Bourgeois-Nicolaos, N., et al., *Maternal vaginal colonisation by Staphylococcus aureus and newborn acquisition at delivery.* Paediatr Perinat Epidemiol, 2010. **24**(5): p. 488-91.
37. Wertheim, H.F., et al., *The role of nasal carriage in Staphylococcus aureus infections.* Lancet Infect Dis, 2005. **5**(12): p. 751-62.
38. Miller, L.G. and B.A. Diep, *Clinical practice: colonization, fomites, and virulence: rethinking the pathogenesis of community-associated methicillin-resistant Staphylococcus aureus infection.* Clin Infect Dis, 2008. **46**(5): p. 752-60.
39. Solberg, C.O., *A study of carriers of Staphylococcus aureus with special regard to quantitative bacterial estimations.* Acta Med Scand Suppl, 1965. **436**: p. 1-96.
40. Williams, R.E., *Healthy carriage of Staphylococcus aureus: its prevalence and importance.* Bacteriol Rev, 1963. **27**: p. 56-71.
41. Kluytmans, J., A. van Belkum, and H. Verbrugh, *Nasal carriage of Staphylococcus aureus: epidemiology, underlying mechanisms, and associated risks.* Clin Microbiol Rev, 1997. **10**(3): p. 505-20.
42. von Eiff, C., et al., *Nasal carriage as a source of Staphylococcus aureus bacteremia. Study Group.* N Engl J Med, 2001. **344**(1): p. 11-6.
43. Kluytmans, J.A. and H.F. Wertheim, *Nasal carriage of Staphylococcus aureus and prevention of nosocomial infections.* Infection, 2005. **33**(1): p. 3-8.
44. Cole, A.M., et al., *Determinants of Staphylococcus aureus nasal carriage.* Clin Diagn Lab Immunol, 2001. **8**(6): p. 1064-9.
45. Zimakoff, J., et al., *Staphylococcus aureus carriage and infections among patients in four haemo- and peritoneal-dialysis centres in Denmark. The Danish Study Group of Peritonitis in Dialysis (DASPID).* J Hosp Infect, 1996. **33**(4): p. 289-300.

46. Immergluck, L.C., et al., *Risk of Skin and Soft Tissue Infections among Children Found to be Staphylococcus aureus MRSA USA300 Carriers.* West J Emerg Med, 2017. **18**(2): p. 201-212.
47. VandenBergh, M.F., et al., *Follow-up of Staphylococcus aureus nasal carriage after 8 years: redefining the persistent carrier state.* J Clin Microbiol, 1999. **37**(10): p. 3133-40.
48. Reta, A., M. Wubie, and G. Mekuria, *Nasal colonization and antimicrobial susceptibility pattern of Staphylococcus aureus among pre-school children in Ethiopia.* BMC Res Notes, 2017. **10**(1): p. 746.
49. Moyo, S.J., et al., *High nasal carriage of methicillin-resistant Staphylococcus aureus among healthy Tanzanian under-5 children.* Microb Drug Resist, 2014. **20**(1): p. 82-8.
50. Eibach, D., et al., *Nasal Carriage of Staphylococcus aureus among Children in the Ashanti Region of Ghana.* PLoS One, 2017. **12**(1): p. e0170320.
51. Usuf, E., et al., *Nasopharyngeal colonization of Gambian infants by Staphylococcus aureus and Streptococcus pneumoniae before the introduction of pneumococcal conjugate vaccines.* New Microbes New Infect, 2016. **10**: p. 13-8.
52. Kwambana, B.A., et al., *Early acquisition and high nasopharyngeal co-colonisation by Streptococcus pneumoniae and three respiratory pathogens amongst Gambian new-borns and infants.* BMC Infect Dis, 2011. **11**: p. 175.
53. Odutola, A., et al., *Comparison of the prevalence of common bacterial pathogens in the oropharynx and nasopharynx of gambian infants.* PLoS One, 2013. **8**(9): p. e75558.
54. Roca, A., et al., *Maternal colonization with Staphylococcus aureus and Group B streptococcus is associated with colonization in newborns.* Clin Microbiol Infect, 2017. **23**(12): p. 974-979.
55. Bojang, A., et al., *Prevalence and risk factors for Staphylococcus aureus nasopharyngeal carriage during a PCV trial.* BMC Infect Dis, 2017. **17**(1): p. 588.
56. Roca, A., et al., *Oral azithromycin given during labour decreases bacterial carriage in the mothers and their offspring: a double-blind randomized trial.* Clin Microbiol Infect, 2016. **22**(6): p. 565 e1-9.
57. Wertheim, H.F., et al., *Nose picking and nasal carriage of Staphylococcus aureus.* Infect Control Hosp Epidemiol, 2006. **27**(8): p. 863-7.
58. Suzuki, K. and K. Tagami, *Role of Nasal Staphylococcus aureus Carriage in Transmission among Contact Athletes.* Int J Sports Med, 2015. **36**(14): p. 1186-91.
59. Sherertz, R.J., et al., *A cloud adult: the Staphylococcus aureus-virus interaction revisited.* Ann Intern Med, 1996. **124**(6): p. 539-47.
60. Bassetti, S., et al., *Dispersal of Staphylococcus aureus into the air associated with a rhinovirus infection.* Infect Control Hosp Epidemiol, 2005. **26**(2): p. 196-203.
61. Jimenez-Truque, N., et al., *Relationship between maternal and neonatal Staphylococcus aureus colonization.* Pediatrics, 2012. **129**(5): p. e1252-9.
62. Benito, D., et al., *Characterization of Staphylococcus aureus strains isolated from faeces of healthy neonates and potential mother-to-infant microbial transmission through breastfeeding.* FEMS Microbiol Ecol, 2015. **91**(3).
63. Fernandez, L., et al., *The human milk microbiota: origin and potential roles in health and disease.* Pharmacol Res, 2013. **69**(1): p. 1-10.
64. Lackey, K.A., et al., *What's Normal? Microbiomes in Human Milk and Infant Feces Are Related to Each Other but Vary Geographically: The INSPIRE Study.* Front Nutr, 2019. **6**: p. 45.
65. Perez, P.F., et al., *Bacterial imprinting of the neonatal immune system: lessons from maternal cells?* Pediatrics, 2007. **119**(3): p. e724-32.
66. Martin R, L.S., Reviriego C, Jimenez E, Marin ML, Olivares M, Boza J, Jimenez J, Fernandez L, Xaus J, *The commensal microflora of human milk: new perspective for food bacteriotherapy and probiotics.* Trends in Food Science & Technology, 2004. **15**(3-4): p. 121-127.
67. Junior, J., et al., *Identification and resistance profile of bacteria isolated on stethoscopes by healthcare professionals: systematic review.* Am J Infect Control, 2020.

68. Donskey, C.J., *Beyond high-touch surfaces: Portable equipment and floors as potential sources of transmission of health care-associated pathogens.* Am J Infect Control, 2019. **47S**: p. A90-A95.
69. Russell, F.M., et al., *As a bacterial culture medium, citrated sheep blood agar is a practical alternative to citrated human blood agar in laboratories of developing countries.* J Clin Microbiol, 2006. **44**(9): p. 3346-51.
70. Harris, L.G., S.J. Foster, and R.G. Richards, *An introduction to Staphylococcus aureus, and techniques for identifying and quantifying S. aureus adhesins in relation to adhesion to biomaterials: review.* Eur Cell Mater, 2002. **4**: p. 39-60.
71. Wang, J.E., et al., *Peptidoglycan and lipoteichoic acid from Staphylococcus aureus induce tumor necrosis factor alpha, interleukin 6 (IL-6), and IL-10 production in both T cells and monocytes in a human whole blood model.* Infect Immun, 2000. **68**(7): p. 3965-70.
72. Kawasaki, A., et al., *Activation of the human complement cascade by bacterial cell walls, peptidoglycans, water-soluble peptidoglycan components, and synthetic muramylpeptides--studies on active components and structural requirements.* Microbiol Immunol, 1987. **31**(6): p. 551-69.
73. Kessler, C.M., E. Nussbaum, and C.U. Tuazon, *Disseminated intravascular coagulation associated with Staphylococcus aureus septicemia is mediated by peptidoglycan-induced platelet aggregation.* J Infect Dis, 1991. **164**(1): p. 101-7.
74. Scully, I.L., et al., *O-Acetylation is essential for functional antibody generation against Staphylococcus aureus capsular polysaccharide.* Hum Vaccin Immunother, 2018. **14**(1): p. 81-84.
75. Lee, J.C., *The prospects for developing a vaccine against Staphylococcus aureus.* Trends Microbiol, 1996. **4**(4): p. 162-6.
76. Foster, T.J. and D. McDevitt, *Surface-associated proteins of Staphylococcus aureus: their possible roles in virulence.* FEMS Microbiol Lett, 1994. **118**(3): p. 199-205.
77. Patti, J.M., et al., *MSCRAMM-mediated adherence of microorganisms to host tissues.* Annu Rev Microbiol, 1994. **48**: p. 585-617.
78. Fries, B.C. and A.K. Varshney, *Bacterial Toxins-Staphylococcal Enterotoxin B.* Microbiol Spectr, 2013. **1**(2).
79. Xu, S.X. and J.K. McCormick, *Staphylococcal superantigens in colonization and disease.* Front Cell Infect Microbiol, 2012. **2**: p. 52.
80. Tuffs, S.W., S.M.M. Haeryfar, and J.K. McCormick, *Manipulation of Innate and Adaptive Immunity by Staphylococcal Superantigens.* Pathogens, 2018. **7**(2).
81. Bhakdi, S. and J. Tranum-Jensen, *Alpha-toxin of Staphylococcus aureus.* Microbiol Rev, 1991. **55**(4): p. 733-51.
82. Diene, S.M., et al., *Human metallo-beta-lactamase enzymes degrade penicillin.* Sci Rep, 2019. **9**(1): p. 12173.
83. Mlynarczyk, A., G. Mlynarczyk, and J. Jeljaszewicz, *The genome of Staphylococcus aureus: a review.* Zentralbl Bakteriol, 1998. **287**(4): p. 277-314.
84. Driebe, E.M., et al., *Using Whole Genome Analysis to Examine Recombination across Diverse Sequence Types of Staphylococcus aureus.* PLoS One, 2015. **10**(7): p. e0130955.
85. Everall, P.H. and P.M. Stacey, *Catalase negative Staphylococcus aureus.* J Med Lab Technol, 1956. **13**(7): p. 489-90.
86. Novick, R.P., *Staphylococcal plasmids and their replication.* Annu Rev Microbiol, 1989. **43**: p. 537-65.
87. Thomas, W.D., Jr. and G.L. Archer, *Identification and cloning of the conjugative transfer region of Staphylococcus aureus plasmid pGO1.* J Bacteriol, 1989. **171**(2): p. 684-91.
88. Kennedy, A.D., et al., *Complete nucleotide sequence analysis of plasmids in strains of Staphylococcus aureus clone USA300 reveals a high level of identity among isolates with closely related core genome sequences.* J Clin Microbiol, 2010. **48**(12): p. 4504-11.
89. McDougal, L.K., et al., *Emergence of resistance among USA300 methicillin-resistant Staphylococcus aureus isolates causing invasive disease in the United States.* Antimicrob Agents Chemother, 2010. **54**(9): p. 3804-11.

90. Ibarz Pavon, A.B. and M.C. Maiden, *Multilocus sequence typing.* Methods Mol Biol, 2009. **551**: p. 129-40.
91. Enright, M.C., et al., *Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of Staphylococcus aureus.* J Clin Microbiol, 2000. **38**(3): p. 1008-15.
92. Maiden, M.C., et al., *Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms.* Proc Natl Acad Sci U S A, 1998. **95**(6): p. 3140-5.
93. Spratt, B.G., *Multilocus sequence typing: molecular typing of bacterial pathogens in an era of rapid DNA sequencing and the internet.* Curr Opin Microbiol, 1999. **2**(3): p. 312-6.
94. Feil, E.J., et al., *How clonal is Staphylococcus aureus?* J Bacteriol, 2003. **185**(11): p. 3307-16.
95. Grundmann, H., et al., *Geographic distribution of Staphylococcus aureus causing invasive infections in Europe: a molecular-epidemiological analysis.* PLoS Med, 2010. **7**(1): p. e1000215.
96. David, M.Z., et al., *Staphylococcus aureus bacteremia at 5 US academic medical centers, 2008-2011: significant geographic variation in community-onset infections.* Clin Infect Dis, 2014. **59**(6): p. 798-807.
97. Gijon, M., et al., *Factors associated with severity in invasive community-acquired Staphylococcus aureus infections in children: a prospective European multicentre study.* Clin Microbiol Infect, 2016. **22**(7): p. 643 e1-6.
98. Egyir, B., et al., *Molecular epidemiology and antimicrobial susceptibility of clinical Staphylococcus aureus from healthcare institutions in Ghana.* PLoS One, 2014. **9**(2): p. e89716.
99. Omuse, G., et al., *Molecular characterization of Staphylococcus aureus isolates from various healthcare institutions in Nairobi, Kenya: a cross sectional study.* Ann Clin Microbiol Antimicrob, 2016. **15**(1): p. 51.
100. Perovic, O., et al., *Prevalence and Trends of Staphylococcus aureus Bacteraemia in Hospitalized Patients in South Africa, 2010 to 2012: Laboratory-Based Surveillance Mapping of Antimicrobial Resistance and Molecular Epidemiology.* PLoS One, 2015. **10**(12): p. e0145429.
101. Kolawole, D.O., et al., *Characterization of colonizing Staphylococcus aureus isolated from surgical wards' patients in a Nigerian university hospital.* PLoS One, 2013. **8**(7): p. e68721.
102. Ebruke, C., et al., *High genetic diversity of Staphylococcus aureus strains colonising the nasopharynx of Gambian villagers before widespread use of pneumococcal conjugate vaccines.* BMC Microbiol, 2016. **16**: p. 38.
103. Senghore, M., et al., *Transmission of Staphylococcus aureus from Humans to Green Monkeys in The Gambia as Revealed by Whole-Genome Sequencing.* Appl Environ Microbiol, 2016. **82**(19): p. 5910-7.
104. Darboe, S., et al., *Prevalence of Panton-Valentine Leukocidin (PVL) and Antimicrobial Resistance in Community-Acquired Clinical Staphylococcus aureus in an Urban Gambian Hospital: A 11-Year Period Retrospective Pilot Study.* Front Cell Infect Microbiol, 2019. **9**: p. 170.
105. Leclercq, R., *Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implications.* Clin Infect Dis, 2002. **34**(4): p. 482-92.
106. Serisier, D.J., *Risks of population antimicrobial resistance associated with chronic macrolide use for inflammatory airway diseases.* Lancet Respir Med, 2013. **1**(3): p. 262-74.
107. Roberts, M.C., et al., *Nomenclature for macrolide and macrolide-lincosamide-streptogramin B resistance determinants.* Antimicrob Agents Chemother, 1999. **43**(12): p. 2823-30.
108. Weisblum, B., *Erythromycin resistance by ribosome modification.* Antimicrob Agents Chemother, 1995. **39**(3): p. 577-85.
109. Fyfe, C., et al., *Resistance to Macrolide Antibiotics in Public Health Pathogens.* Cold Spring Harb Perspect Med, 2016. **6**(10).

110. Ross, J.I., et al., *Inducible erythromycin resistance in staphylococci is encoded by a member of the ATP-binding transport super-gene family.* Mol Microbiol, 1990. **4**(7): p. 1207-14.
111. Bojang, E., et al., *Short-term increase in prevalence of nasopharyngeal carriage of macrolide-resistant Staphylococcus aureus following mass drug administration with azithromycin for trachoma control.* BMC Microbiol, 2017. **17**(1): p. 75.
112. Burmeister, A.R., *Horizontal Gene Transfer.* Evol Med Public Health, 2015. **2015**(1): p. 193-4.
113. Daubin, V. and G.J. Szollosi, *Horizontal Gene Transfer and the History of Life.* Cold Spring Harb Perspect Biol, 2016. **8**(4): p. a018036.
114. Parte, A.C., *LPSN - List of Prokaryotic names with Standing in Nomenclature (bacterio.net), 20 years on.* Int J Syst Evol Microbiol, 2018. **68**(6): p. 1825-1829.
115. von Eiff, C., G. Peters, and C. Heilmann, *Pathogenesis of infections due to coagulase-negative staphylococci.* Lancet Infect Dis, 2002. **2**(11): p. 677-85.
116. Choi, S.H., et al., *Clinical significance of Staphylococcus saprophyticus identified on blood culture in a tertiary care hospital.* Diagn Microbiol Infect Dis, 2006. **56**(3): p. 337-9.
117. Otto, M., *Staphylococcus epidermidis--the 'accidental' pathogen.* Nat Rev Microbiol, 2009. **7**(8): p. 555-67.
118. Becker, K., C. Heilmann, and G. Peters, *Coagulase-negative staphylococci.* Clin Microbiol Rev, 2014. **27**(4): p. 870-926.
119. Cetin, E.S., et al., *Distribution of genes encoding resistance to macrolides, lincosamides and streptogramins among clinical staphylococcal isolates in a Turkish university hospital.* J Microbiol Immunol Infect, 2010. **43**(6): p. 524-9.
120. Gatermann, S.G., T. Koschinski, and S. Friedrich, *Distribution and expression of macrolide resistance genes in coagulase-negative staphylococci.* Clin Microbiol Infect, 2007. **13**(8): p. 777-81.
121. Le Bouter, A., R. Leclercq, and V. Cattoir, *Molecular basis of resistance to macrolides, lincosamides and streptogramins in Staphylococcus saprophyticus clinical isolates.* Int J Antimicrob Agents, 2011. **37**(2): p. 118-23.
122. Jeric, P.E., et al., *Survey of molecular determinants in Gram-positive cocci isolated from hospital settings in Argentina.* J Infect Dev Ctries, 2007. **1**(3): p. 275-83.
123. Deplano, A., et al., *National surveillance of Staphylococcus epidermidis recovered from bloodstream infections in Belgian hospitals.* J Antimicrob Chemother, 2016. **71**(7): p. 1815-9.
124. Juda, M., B. Chudzik-Rzad, and A. Malm, *The prevalence of genotypes that determine resistance to macrolides, lincosamides, and streptogramins B compared with spiramycin susceptibility among erythromycin-resistant Staphylococcus epidermidis.* Mem Inst Oswaldo Cruz, 2016. **111**(3): p. 155-60.
125. Bakheit, A.H., B.M. Al-Hadiya, and A.A. Abd-Elgalil, *Azithromycin.* Profiles Drug Subst Excip Relat Methodol, 2014. **39**: p. 1-40.
126. Chico, R.M. and D. Chandramohan, *Azithromycin plus chloroquine: combination therapy for protection against malaria and sexually transmitted infections in pregnancy.* Expert Opin Drug Metab Toxicol, 2011. **7**(9): p. 1153-67.
127. Jelic, D. and R. Antolovic, *From Erythromycin to Azithromycin and New Potential Ribosome-Binding Antimicrobials.* Antibiotics (Basel), 2016. **5**(3).
128. Harding-Esch, E.M., et al., *Mass treatment with azithromycin for trachoma: when is one round enough? Results from the PRET Trial in the Gambia.* PLoS Negl Trop Dis, 2013. **7**(6): p. e2115.
129. Yohannan, J., et al., *Can we stop mass drug administration prior to 3 annual rounds in communities with low prevalence of trachoma?: PRET Ziada trial results.* JAMA Ophthalmol, 2013. **131**(4): p. 431-6.
130. Gebre, T., et al., *Comparison of annual versus twice-yearly mass azithromycin treatment for hyperendemic trachoma in Ethiopia: a cluster-randomised trial.* Lancet, 2012. **379**(9811): p. 143-51.

131. Schemann, J.F., et al., *Longitudinal evaluation of three azithromycin distribution strategies for treatment of trachoma in a sub-Saharan African country, Mali.* Acta Trop, 2007. **101**(1): p. 40-53.
132. Porco, T.C., et al., *Effect of mass distribution of azithromycin for trachoma control on overall mortality in Ethiopian children: a randomized trial.* JAMA, 2009. **302**(9): p. 962-8.
133. Burr, S.E., et al., *Mass administration of azithromycin and Streptococcus pneumoniae carriage: cross-sectional surveys in the Gambia.* Bull World Health Organ, 2014. **92**(7): p. 490-8.
134. Unger, H.W., et al., *Impact of intermittent preventive treatment in pregnancy with azithromycin-containing regimens on maternal nasopharyngeal carriage and antibiotic sensitivity of Streptococcus pneumoniae, Haemophilus influenzae, and Staphylococcus aureus: a cross-sectional survey at delivery.* J Clin Microbiol, 2015. **53**(4): p. 1317-23.
135. Luntamo, M., et al., *The effect of antenatal monthly sulphadoxine-pyrimethamine, alone or with azithromycin, on foetal and neonatal growth faltering in Malawi: a randomised controlled trial.* Trop Med Int Health, 2013. **18**(4): p. 386-97.
136. Hare, K.M., et al., *Nasopharyngeal carriage and macrolide resistance in Indigenous children with bronchiectasis randomized to long-term azithromycin or placebo.* Eur J Clin Microbiol Infect Dis, 2015. **34**(11): p. 2275-85.
137. Hansen, C.R., et al., *Long-term, low-dose azithromycin treatment reduces the incidence but increases macrolide resistance in Staphylococcus aureus in Danish CF patients.* J Cyst Fibros, 2009. **8**(1): p. 58-62.
138. Roca, A., et al., *Prevention of bacterial infections in the newborn by pre-delivery administration of azithromycin: Study protocol of a randomized efficacy trial.* BMC Pregnancy Childbirth, 2015. **15**: p. 302.
139. O'Brien, K.L., H. Nohynek, and G. World Health Organization Pneumococcal Vaccine Trials Carriage Working, *Report from a WHO Working Group: standard method for detecting upper respiratory carriage of Streptococcus pneumoniae.* Pediatr Infect Dis J, 2003. **22**(2): p. e1-11.
140. Oluwalana, C., et al., *Azithromycin in Labor Lowers Clinical Infections in Mothers and Newborns: A Double-Blind Trial.* Pediatrics, 2017. **139**(2).
141. CLSI, *Performance Standards for Antimicrobial Susceptibility Testing*. 2017. p. 224.
142. Bankevich, A., et al., *SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing.* J Comput Biol, 2012. **19**(5): p. 455-77.
143. Wick, R.R., et al., *Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads.* PLoS Comput Biol, 2017. **13**(6): p. e1005595.
144. Seemann, T., *Prokka: rapid prokaryotic genome annotation.* Bioinformatics, 2014. **30**(14): p. 2068-9.
145. Hisatsune, J., et al., *Complete Genome Sequence of Systemically Disseminated Sequence Type 8 Staphylococcal Cassette Chromosome mec Type IVl Community-Acquired Methicillin-Resistant Staphylococcus aureus.* Genome Announc, 2017. **5**(35).
146. Carver, T.J., et al., *ACT: the Artemis Comparison Tool.* Bioinformatics, 2005. **21**(16): p. 3422-3.
147. Nguyen, L.T., et al., *IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies.* Mol Biol Evol, 2015. **32**(1): p. 268-74.
148. Felsenstein, J., *Evolutionary trees from DNA sequences: a maximum likelihood approach.* J Mol Evol, 1981. **17**(6): p. 368-76.
149. Hasegawa, M., H. Kishino, and T. Yano, *Dating of the human-ape splitting by a molecular clock of mitochondrial DNA.* J Mol Evol, 1985. **22**(2): p. 160-74.
150. Hoang, D.T., et al., *UFBoot2: Improving the Ultrafast Bootstrap Approximation.* Mol Biol Evol, 2018. **35**(2): p. 518-522.
151. Guerillot, R., et al., *Convergent Evolution Driven by Rifampin Exacerbates the Global Burden of Drug-Resistant Staphylococcus aureus.* mSphere, 2018. **3**(1).
152. Petit, R.A., 3rd and T.D. Read, *Staphylococcus aureus viewed from the perspective of 40,000+ genomes.* PeerJ, 2018. **6**: p. e5261.

153. Yu, G., et al., *Two Methods for Mapping and Visualizing Associated Data on Phylogeny Using Ggtree.* Mol Biol Evol, 2018. **35**(12): p. 3041-3043.
154. Bojang, A., et al., *Long-term Impact of Oral Azithromycin Taken by Gambian Women During Labor on Prevalence and Antibiotic Susceptibility of Streptococcus pneumoniae and Staphylococcus aureus in Their Infants: Follow-up of a Randomized Clinical Trial.* Clin Infect Dis, 2018. **67**(8): p. 1191-1197.
155. Brown-Jaque, M., W. Calero-Caceres, and M. Muniesa, *Transfer of antibiotic-resistance genes via phage-related mobile elements.* Plasmid, 2015. **79**: p. 1-7.
156. Lerminiaux, N.A. and A.D.S. Cameron, *Horizontal transfer of antibiotic resistance genes in clinical environments.* Can J Microbiol, 2019. **65**(1): p. 34-44.
157. Deurenberg, R.H. and E.E. Stobberingh, *The evolution of Staphylococcus aureus.* Infect Genet Evol, 2008. **8**(6): p. 747-63.
158. Bojang, A., et al., *Genomic investigation of Staphylococcus aureus recovered from Gambian women and newborns following an oral dose of intra-partum azithromycin.* J Antimicrob Chemother, 2019. **74**(11): p. 3170-3178.
159. Ojo, K.K., et al., *Staphylococcus efflux msr(A) gene characterized in Streptococcus, Enterococcus, Corynebacterium, and Pseudomonas isolates.* Antimicrob Agents Chemother, 2006. **50**(3): p. 1089-91.
160. Nawaz, M.S., A.A. Khan, and C.E. Cerniglia, *Detection of erythromycin resistant methylase gene by the polymerase chain reaction.* Mol Cell Probes, 1997. **11**(5): p. 317-22.
161. Jensen, L.B., N. Frimodt-Moller, and F.M. Aarestrup, *Presence of erm gene classes in gram-positive bacteria of animal and human origin in Denmark.* FEMS Microbiol Lett, 1999. **170**(1): p. 151-8.
162. Dziri, R., et al., *High prevalence of Staphylococcus haemolyticus and Staphylococcus saprophyticus in environmental samples of a Tunisian hospital.* Diagn Microbiol Infect Dis, 2016. **85**(2): p. 136-40.
163. Mack, I., et al., *Antimicrobial Resistance Following Azithromycin Mass Drug Administration: Potential Surveillance Strategies to Assess Public Health Impact.* Clin Infect Dis, 2019.